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THE UNIVERSITY OF ALBERTA

PHYSIOLOGICAL RESPONSE OF SODIUM-DEPENDENT *AZOTOBACTER CHROOCOCCUM* STRAIN 184 TO OXYGEN STRESS

by

Shailaja Shivprasad

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF MICROBIOLOGY

EDMONTON, ALBERTA

SPRING, 1991

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To my mother

Without whose help life as a graduate student and a mother would have been much more difficult than it really was. She stayed with me all through the duration of my program after the birth of my son and looked after him while I was away at school. I could not have got a better caregiver for Aditya. Thank you, Mummy.

ABSTRACT

The physiological response of Na^+-dependent *Azotobacter chroococcum* strain 184 to oxygen stress was investigated in this study.

The difficulty in recovering strain 184 quantitatively on solid plating media was partially reversed by charcoal, a free radical scavenger. Its sensitivity to H_2O_2 and the subsequent discovery of the lack of detectable catalase activity warranted an investigation into the optimal O_2 requirements of the organism. At low population densities the organism grew under microaerobic conditions in nitrogen-free medium, whereas at higher population densities it demonstrated a preference for an aerobic environment. Further studies demonstrated a preference for lower oxygen tensions with organic acids as carbon source and higher oxygen tensions with carbohydrates. Despite its microaerophilic characteristics the organism demonstrated aeroadaptative capabilities which were further investigated.

Under nitrogen-fixing conditions the organism produced catechol which was polymerized to a melanin. An organism is more oxygen stressed under nitrogen-fixing conditions than in the presence of ammonium. Catechol and melanin production were accelerated in the presence of high iron and high aeration, conditions which would promote an increase in the intracellular production of oxygen radicals. Charcoal and benzoic acid, free radical scavengers, inhibited melanization. Histidine, a free radical scavenger inhibited catechol formation. In the presence of copper there was a total loss of catalase and peroxidase activity, and melanization was accelerated. Based on these results a role for catechol and melanin formation in aeroadaptation is proposed.

Catechol formation was repressed by ammonium at concentrations which also repressed nitrogenase in strain 184. The glutamine analog methionine sulfoximine which reverses the repressive effect of ammonium on nitrogenase, also reversed its effect on catechol formation implicating a similar regulatory mechanism in both systems.

A genomic library of strain 184 was constructed in *Escherichia coli* using the cosmid cloning vector pLAFR3. A melanin synthesizing *E. coli* clone was isolated and the 22 kb insert was subcloned as a 1.87 kb fragment into pUC119. Tn5 mutagenesis of the cloned gene in *E. coli* was carried out using Tn5*tac*1. This resulted in the isolation of mutations in the *ppo* structural gene which would be useful in isolating a *mel*$^-$ mutant of strain 184 and in studying the role of melanin in aeroadaptation.

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ABBREVIATIONS

A_{260}	absorbance at 260 nm
A_{280}	absorbance at 280 nm
A_{400}	absorbance at 400 nm
A_{503}	absorbance at 503 nm
ACE	sodium acetate (0.2M in 90% v/v ethanol)
ATCC	American Type Culture Collection
BBGN	Burk's buffer containing glucose and an ammonium source.
CCC	covalently closed circular
DNA	deoxyribonucleic acid
DHN	dihydroxynaphthalene
DOPA	dihydroxyphenylalanine
EDDHA	ethylenediamine di-(o-hydroxyphenyl acetic acid)
EDTA	ethylenediaminetetraacetic acid
K	1000 daltons
kb	kilo base
MSX	methionine sulfoximine
NCIB	National Collection of Industrial Bacteria
nm	nanometre
NTA	nitrilotriacetic acid
OD_{620}	optical density at 620 nm
ODPX	o-dianisidine peroxidase
PDPX	p-phenylenediamine peroxidase
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
SOD	superoxide dismutase
TLC	thin layer chromatography
Tris	Tris(hydroxymethyl)amino methane
ppo	polyphenol oxidase

CHAPTER 1

General Introduction

1.1 OXYGEN: BOON AND BANE

Molecular oxygen is used by living organisms in two ways: as an electron acceptor and as a source of oxygen atoms incorporated into various substrates via oxygenases (Morris, 1984). Its most important function is due to its ability to act as an oxidant, this property being exploited by all organisms capable of aerobic respiration for the purpose of free energy conservation (Morris, 1984).

All respiring organisms are, unfortunately, in a dilemma, because oxygen, which supports their very existence, is also a toxic substance and organisms can survive in its presence only because of the presence of an elaborate system of defenses (Fridovich, 1975) which appear adequate under ordinary conditions. Stouthamer et al. (1979) describe aerobic bacteria as "bacteria able to respire with oxygen, unable to grow in the absence of an external hydrogen acceptor and thus unable to obtain the energy required for growth from fermentation reactions". Organisms not utilizing oxygen are called anaerobes and are of two types: obligate anaerobes, which live in the absence of oxygen, and are killed by exposure to oxygen since they do not possess the necessary defenses required for aerobic growth, and aerotolerant anaerobes which are not killed by exposure to oxygen (Stanier et al. 1976). Stanier et al. (1976) describe different types of aerobes as follows, "many obligate aerobes cannot grow at oxygen concentrations greater than atmospheric (> 20% O_2). Indeed, some require O_2 concentrations considerably lower than atmospheric (2 to 10% v/v) in order to grow. Aerobic bacteria which show such oxygen sensitivity under all growth conditions, are called microaerophiles". Stouthamer et al. (1979) describe microaerophiles in the following way, "aerobic or facultative anaerobic bacteria, which under aerobic conditions use O_2 as hydrogen acceptor in a respiratory way of energy production and show optimal growth at low dissolved oxygen tensions".

1.1.1 The generation of toxic oxygen radicals: the Fenton reaction

The reduction of O_2 to $2H_2O$ requires four electrons. Since oxygen exhibits a preference for univalent pathways of reduction, superoxide ($O_2^{\cdot-}$) and the hydroxy radical ($\cdot OH$) are possible intermediates (Fridovich, 1975) in this reduction in addition to hydrogen peroxide. Thus partial reduction of the dioxygen molecule results in the

formation of hazardous by-products which are life threatening (Fridovich, 1975). The most damaging effect of $O_2^{\cdot}$ results when hydrogen peroxide reacts with the superoxide radical in the presence of iron salts resulting in the formation of the powerfully reactive hydroxyl radical. This reaction is the Fenton reaction (Dunford, 1987):

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + HO_2 + H^+$$
$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {\cdot}OH + OH^-$$
$$Fe^{3+} + HO_2 \rightarrow Fe^{2+} + O_2 + H^+$$

Since the solubility of ferric iron is low at pH 7, and both the ferrous ion and ferric ion tend to form coordination complexes, the probability of finding free ferric or ferrous ions in solution is very low (Dunford, 1987). It has been suggested that the intracellular iron pool consists of iron bound to ligands such as ADP, ATP, or citrate (Halliwell and Gutteridge, 1985), NADH and NADPH (Rowley and Halliwell, 1982), and DNA which are thought to promote the Fenton reaction in the cell (Dunford, 1987).

1.1.2 Alternate sources of oxygen radicals in aerobic systems

In addition to the univalent reduction of oxygen, several spontaneous autooxidations and enzymatic oxidations also generate superoxides. Among these are the autooxidations of reduced flavins, hydroquinones, and catecholamines and the oxidations of reduced ferredoxins and hemeproteins (Fridovich, 1975; Robson and Postgate, 1980). The enzymatic reactions which generate superoxides are due to the aerobic action of xanthine oxidase, aldehyde oxidase, dihydroorotic dehydrogenase and several flavin dehydrogenases (Fridovich, 1975). Because the production of toxic oxygen radicals is inevitable in respiring cells, they have developed both enzymatic and non-enzymatic defenses to remove these noxious agents from the interior of the cell.

1.1.3 Damage by hydroxyl radicals

Hydroxyl radicals are known to fragment polysaccharides like hyaluronic acid and they are also involved in lipid peroxidation (Wolff et al. 1986). Hydroxyl radicals can initiate the process of lipid peroxidation in membranes (Halliwell and Gutteridge, 1988) resulting in successive reactions as follows:

$$\text{lipid-H} + {\cdot}OH \rightarrow \text{lipid}{\cdot} + H_2O$$

Under aerobic conditions, the subsequent reaction would be:

$$\text{lipid}{\cdot} + O_2 \rightarrow \text{lipid}O_2{\cdot}$$

The peroxy radical can abstract further H atoms propagating a chain reaction of lipid peroxidation as follows:

$$\text{lipid}O_2{\cdot} + \text{lipid-H} \rightarrow \text{lipid}O_2H + \text{lipid}{\cdot}$$

In addition to damage to lipids, hydroxyl radicals can modify amino acid residues, resulting in changes in enzymatic activity, and also cause protein fragmentation

(Wolff et al. 1986). The modified proteins are more susceptible to enzymatic hydrolysis as has been seen with glutamine synthetase where the oxidized protein is more susceptible to the action of different proteinases (Wolff et al. 1986). In addition, hydroxyl radicals can hydroxylate aromatic molecules like phenol and salicylate, this reaction being used as an assay system to demonstrate the generation of hydroxyl radicals (Richmond et al. 1981).

Damage to DNA is caused by hyperbaric oxygen, gamma radiation, H_2O_2, and $O_2^{\cdot}$-generating drugs. The oxygen radical may attack the DNA either at the sugar or at the base (Imlay and Linn, 1988) resulting in single stranded DNA breaks. This effect of the hydroxyl radical on DNA is being exploited in the hydroxyl radical footprinting method for the study of protein interaction with DNA (Tullius et al. 1989). Since DNA binds metal, it is an immediate target for oxidative damage via the Fenton reaction (Imlay and Linn, 1988).

1.2 DEFENSES AGAINST TOXIC OXYGEN RADICALS

1.2.1 Enzymatic defenses

Superoxide dismutases (SOD) are oxidoreductases which catalyze the dismutation of the superoxide radical to O_2 and hydrogen peroxide by the reaction (Beauchamp and Fridovich, 1971):

$$O_2^{\cdot} + O_2^{\cdot} + 2H^+ \rightarrow H_2O_2 + O_2$$

Hydrogen peroxide, which is also a threat to the chemical integrity of the cell is eliminated by the action of catalases and peroxidases (Fridovich, 1983), where peroxidases catalyze the oxidation of organic compounds by H_2O_2 via the following reaction (Lück, 1963):

$$AH_2 + H_2O_2 \rightarrow 2H_2O + A$$

Superoxide dismutases, catalases and peroxidases comprise a defensive team in respiring cells and are mutually supportive, because, superoxides, which inactivate catalases and peroxidases by converting the resting ferric enzyme to the poorly active ferro-oxy form, are protected by SOD against this inactivation. In turn, SOD which is inactivated by H_2O_2 is protected by catalases and peroxidases (Fridovich, 1983).

Further, the enzymes cytochrome oxidase, tyrosinase and ascorbic oxidase are also thought to be involved in partially protecting aerobic cells by avoiding the formation of activated oxygen products (Sichel et al. 1987).

1.2.2 Non-enzymatic defense system

Among the nonenzymatic antioxidants are uric acids and urates (Ames et al. 1981), vitamin C and plasma proteins (Wayner et al. 1985), vitamin E (Burton and Ingold, 1986), β-carotene (Burton and Ingold, 1984), and melanin (Sichel et al. 1987). The principal mode of action of these non-enzymatic protectants is due to a displacement reaction (Dunford, 1987) in which a reactive free radical (R'·) is converted into a less reactive one (R·), as shown in the following reaction:

$$R'\cdot + RH \rightarrow R'H + R\cdot$$

Among the other defenses, certain bacteria like *Peptostreptococcus anaerobius* have less obvious mechanisms of self protection which involve the reduction of O_2 to water with minimal formation of $O_2^{\cdot}$ or H_2O_2 (Hoshino et al. 1978), or organisms which have DNA repair systems which help to minimize the actual damage that may be done by a reagent like H_2O_2 (Carlsson and Carpenter, 1980). Both bacteria and mammalian cells contain enzymes which excise damaged sugars and bases from DNA. For example, it has been demonstrated that mutants of *E. coli* defective in DNA polymerase I, genetic recombination or sugar fragment excision are very sensitive to oxidative stress (Ananthaswamy and Eisenstark, 1977). Hydroxyl radical scavengers such as mannitol, histidine and glucose have high iron-binding affinities and are thought to be involved in protecting the target molecule like DNA (Rozenberg-Arska et al. 1985) from damage mediated by the Fenton reaction due to this metal-binding property (Gutteridge, 1987), by transferring the damage to themselves (Gutteridge, 1984). In most extracellular fluids, it is thought that the major antioxidant defense is to keep metal ions from participating in free radical reactions, this role being played by transferrin, albumin, haptoglobin, haemopexin and uric acid (Halliwell and Gutteridge, 1986).

1.3 MELANINS

1.3.1 Distribution and chemical composition

Melanins are dark brown to black pigments found in animals, plants and microorganisms (Bell and Wheeler, 1986). Among bacteria their presence has been reported in *Streptomyces* spp. (Arai and Mikami, 1972), *Vibrio cholerae* (Ivins and Holmes, 1980), *Azospirillum brasilense* (Sadasivan and Neyra, 1987), *Rhizobium* spp. (Beynon et al. 1980), *Mycobacterium smegmatis* (Prabhakaran et al. 1968), and *Micrococcus melaninogenerans* (Mung and Kondrat'eva, 1981). They are known to enhance the survival and competitive abilities of the producing species, although they are not essential for growth and development. They are produced by the oxidative

polymerization of phenolic compounds such as catechol, tyrosine or dihydroxyphenylalanine (DOPA), γ-glutaminyl-3,4-dihydroxybenzene (GHB) and dihydroxynaphthelene (DHN) due to the action of a polyphenol oxidase (tyrosinase), resulting in the formation of catechol melanins, DOPA melanins, GDHB melanins and DHN melanins, respectively (Bell and Wheeler, 1986) (Fig. 1.1).

1.3.2 Tyrosinases

Tyrosinases catalyze two types of reactions: the orthohydroxylation of monophenols to diphenols, the cresolase activity, and the dehydrogenation of catechols to o-quinones, the catecholase activity (Lerch and Ettlinger, 1972). Since molecular oxygen is the source of oxygen for the hydroxylation, the enzyme is also called a "mixed function oxidase" when it acts on monophenols (Lerch and Ettlinger, 1972). The active site of the enzyme contains two copper atoms bonded to histidine groups (Wigfield and Goltz, 1989). The primary role of the enzyme in living systems is to produce melanin (Hearing and Ekkel, 1976) and the reaction catalyzed by the enzyme is the rate-controlling step in the biosynthetic pathway (Hearing and Jiminez, 1987), the subsequent steps being non enzymatic (della Cioppa et al. 1990).

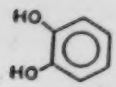
The enzyme has been purified from several sources (Pomerantz and Murthy, 1974; Lerch and Ettlinger, 1972; Duckworth and Coleman, 1970; Pomerantz, 1963) and there is considerable variation in molecular weights among the different sources.

1.3.3 Properties of melanin

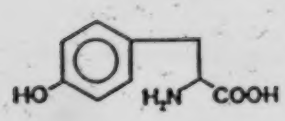
The black colour of melanin pigments is due to their absorption of all visible wavelengths. Absorption by black melanin pigments increases in the UV region and decreases as the wavelength increases up to the far-red region (Bell and Wheeler, 1986). The melanin polymer contains mixtures of quinone, hydroquinone, and semiquinone moieties. This accounts for the light absorption by the pigment, the stabilization of the semiquinone free radicals in the polymer and the ability of the pigment to act as a "sponge" for other free radicals which result due to environmental stress (Bell and Wheeler, 1986). Melanins can also act as reductants or oxidants, and it is this property which is used to identify a pigment as a melanin, viz. oxidation by H_2O_2 and reduction by silver ions (Bell and Wheeler, 1986).

1.3.4 Protective role of melanin

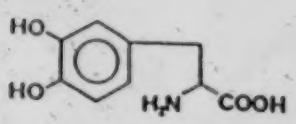
Mason et al. (1960) attribute the protective effect of the pigment to tissues, against reducing and oxidizing conditions, to the ability of melanin to trap free radicals and to act as an electron exchange polymer. Geremia et al. (1984) suggest that the free radical scavenging action of melanins could play an important role in protecting cells from the



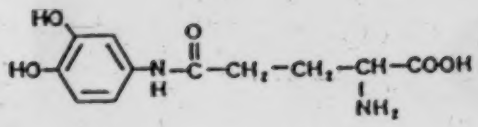
catechol



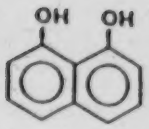
tyrosine



dihydroxyphenylalanine (DOPA)



γ-glutaminyl-3,4-dihydroxybenzene(GDHB)



1,8-dihydroxynaphthalene (DHN)

Fig. 1.1 Phenolic precursors of melanin. Adapted from Bell and Wheeler (1986).

toxic effects of $O_2^{\cdot-}$. Free radicals can oxidize polyunsaturated fatty acids of cellular membranes and thiol groups of free amino acids, and cause other alterations to DNA and enzyme proteins. Scalia et al. (1990) demonstrated that the susceptibility to lipid peroxidation, *in vitro*, of liver tissues containing melanin, was lower than that in unpigmented tissue and therefore suggested that melanins could reduce lipoperoxidation in pigmented tissue. Along similar lines, Sakina et al. (1980) demonstrated the inhibition of lipid peroxidation of the pigmented epithelium of the eye by melanoprotein granules.

DOPA melanin can act as a cation exchanger (White, 1958). Thus melanins can bind significant amounts of metal ions like iron, copper, manganese, nickel, cobalt and magnesium (Bruenger et al. 1967; Potts, 1976). Since melanin is able to reduce free ferric ions to the ferrous form, and ferrous ions bound to melanin are unable to participate efficiently in Fenton-type reactions (Pilas et al. 1988), melanins are thought to play an important role in protection from oxidative stress.

Melanized spores of fungi are resistant to killing by ultraviolet light. Melanins absorb the radiation and dissipate the energy primarily by undergoing reversible increases in free radicals (Bell and Wheeler, 1986). In man and other verterbrates, melanized skin and hair serve as sources of camouflage (Morison, 1985) and one of the major functions of melanin in the skin is photoprotection (Korytowski et al. 1985). The "ink" released by squid and octopus, which consists of a fine suspension of melanin granules, acts as screens against predators (Mimura et al. 1982). In invertebrates, melanins function in camouflage and also as part of the immune response against microorganisms (Bell and Wheeler, 1986). In fungi, melanins are important in increasing resistance to microbial attack, particularly against the hydrolytic enzymes produced by the microorganism (Bloomfield and Alexander, 1967). Melanins are required for the penetration of rice, cucumber, and bean by appresoria of *Pyricularia oryzae*, *Colletotrichum lagenarium*, and *C. lindemuthianium*, respectively (Bell and Wheeler, 1986), thus increasing the virulence of the pathogenic organism. There have been occasional instances where melanins have antibiotic effect against other organisms, e.g. melanins from cell walls of *Phellinus weiri* act as antibiotics to other organisms in culture assays (Li, 1983).

Among bacteria no definite role for melanin has been described, although Hawkins and Johnston (1988) suggest that it may have a role in nitrogen fixation. The same authors speculate on other functions of melanins in *Rhizobium*, viz., to detoxify phenolic toxins which are present in the nodules of wild species of *Phaseolus*, and secondly, since senescing root nodules excrete toxic phenolic compounds, the tyrosinase may have a role in protecting the organism from these compounds.

1.3.5 Melanin and SOD activity

Korytowski et al. (1985) report a pseudo-superoxide dismutase activity of melanin, where about 20% of the reaction between superoxide and DOPA-melanin occurs via reduction of superoxide to hydrogen peroxide, and approximately 80% of the reaction occurs via oxidation to molecular oxygen. In a study by Sichel et al. (1987) and Geremia et al. (1984) an inverse relationship between SOD and melanin in various tissues was observed. Due to the ability of melanins to interact with hydroxyl radicals and superoxides (Sarna et al. 1986; Korytowski et al. 1986) and its ability to bind ferrous ions and therefore make it unavailable in the Fenton reaction, melanins are considered to be important in protecting cells from oxidative stress (Scalia et al. 1990).

1.3.6 Regulation of melanin formation

Although much information on the regulatory aspects of melanin formation is not available, some work has been done on this aspect in *Streptomyces* spp. and in *Rhizobium leguminosarum*. In *Streptomyces glaucescens*, production of melanin is induced by different amino acids, like L-methionine, L-leucine and L-phenylalanine (Katz and Betancourt, 1988). Studies on *Streptomyces antibioticus* demonstrated that L-methionine induced tyrosinase in this organism and that both transcriptional and translational events were required for tyrosinase induction (Katz and Betancourt, 1988). In many strains of this genus, melanin production is an unstable property (Katz et al. 1983) and is thought to be either plasmid encoded or chromosomal (Gregory and Huang, 1964; Crameri et al. 1982). The tyrosinase gene from *Streptomyces antibioticus* has been cloned in *E. coli* (Altenbuchner, 1988; della Cioppa et al. 1990; Tseng et al. 1990) and in *Streptomyces lividans* (Katz et al. 1983). In addition, production of melanin in *Streptomyces* species requires the presence of another gene product, ORF 438, which is thought to function as a metallothionein-like protein that delivers copper to apotyrosinase (Bernan et al. 1985; Lee et al. 1988). In *Rhizobium leguminosarum* biovar *phaseoli*, melanin synthesis is a plasmid-encoded property which is activated by NifA (Hawkins and Johnston, 1988), the activator of *nif* genes.

1.4 IRON REQUIREMENT AND RESPONSE OF MICROORGANISMS TO IRON LIMITATION

Iron is an essential element required for the growth of almost all organisms. It is used for transport (hemoglobin) and storage (myoglobin) of oxygen, and in cytochromes and cytochrome oxidase for the utilization of oxygen for respiration (Halliwell and Gutteridge, 1988). It is also an essential component of several enzymes, such as

nitrogenase (Robson and Postgate, 1980), aconitase, and the protective enzymes catalase and superoxide dismutase (Halliwell and Gutteridge, 1988).

Although iron is the fourth most abundant element in the environment, its low solubility limits its availability to microorganisms (Neilands, 1974). At pH 7, the solubility of Fe^{2+} is 10^{-1} M and that of Fe^{3+}, 10^{-18} M (Spiro and Saltman, 1974). Under aerobic conditions the ferric form is predominant and therefore the availability of iron to cells is limited (Spiro and Saltman, 1974).

In response to iron limitations, microorganisms produce low molecular weight iron chelators called siderophores (Neilands, 1981) which specifically chelate iron and solubilize it, making it available to the organism. Siderophores are classified into two main groups, the catecholates and the hydroxamates. Examples of the former class are enterobactin, produced by *Salmonella typhimurium* and *E. coli* (Neilands, 1981), and of the latter class, ferrioxamines, produced by actinomycetes.

When stressed for iron, microbes often excrete phenolic iron-binding substances such as 2,3-Dihydroxybenzoyl glycine, by *Bacillus subtilis* (Ito and Neilands, 1958), salicylic acid, 3,4-Dihydroxybenzoic acids and catechol by *Mycobacterium smegmatis* (Ratledge and Winder, 1966) and 2,3-Dihydroxybenzoic acid and 2-*N*,6-*N*-di-(2,3-Dihydroxybenzoyl)-L-lysine by iron-deficient *Azotobacter vinelandii* (Corbin and Bulen, 1969). 3,4-Dihydroxybenzoic acid which is produced by *Azomonas macrocytogenes* and *Azotobacter paspali* were found to solubilize iron from minerals and were suggested to be involved in facilitating low-affinity iron uptake in these organisms (Collinson et al. 1986). The production of these compounds is not repressed by high levels of iron in the growth medium, and therefore these compounds do not classify as siderophores which may be defined as, "low molecular weight (500-1000 daltons) virtually ferric specific ligands, the biosynthesis of which is carefully regulated by iron and the function of which is to supply iron to the cell". (Neilands, 1981).

Organisms take great care to keep iron bound to proteins wherever possible so that it restricts the availability to inhibit bacterial growth (Weinberg, 1984) and also because the availability of iron stimulates damaging free radical reactions, resulting in the formation of superoxides and hydrogen peroxide (Halliwell and Gutteridge, 1988). Metal-chelating agents that bind with non-protein-bound metal ions and prevent them from participating in such reactions have therapeutic potential in several diseases such as inflammatory joint diseases, and post-trauma degeneration of the brain and spinal cord (Halliwell and Gutteridge, 1988).

1.5 MICROAEROPHILES

Microaerophiles are organisms for which oxygen is both beneficial and deleterious. Examples of microaerophiles are the saprophytic *Spirillum volutans* (Wells and Krieg, 1965); the magnetic bacteria *Aquaspirillum magnetotacticum* (Maratea and Blakemore, 1981); aquatic chemolitotrophs such as *Gallionella* and *Beggiatoa* species (Jorgensen and Revsbech, 1983); pathogenic organisms such as *Campylobacter* species (Smibert, 1978) and *Treponema pallidum* (Krieg and Hoffman, 1986); and nitrogen-fixing organisms viz. *Azospirillum* spp. (Dobereiner and Day, 1976), *Rhizobium* and *Bradyrhizobium* spp. (Keister, 1975; Kurtz and LaRue, 1975; McComb et al. 1975; Pagan et al. 1975).

Several theories have been put forward for explaining the cause of microaerophily. Among these are: sensitivity to toxic forms of oxygen in culture media which are generated when culture media are illuminated (Hoffman et al. 1979), excretion of toxic forms of oxygen by microaerophiles themselves (Krieg and Hoffman, 1986), low respiratory rates, as seen for *Azospirillum* spp. which lack respiratory protection for the nitrogenase complex (Krieg and Hoffman, 1986), and lack of protective enzymes, viz. catalase (Maratea and Blakemore, 1981, Padgett et al. 1982). In addition it has also been suggested that microaerophiles may have unusually oxygen-sensitive sites as cell constituents (Krieg and Hoffman, 1986), such as the periplasm and outer membrane (Logan and Trust, 1982), membrane-bound cytochromes (Hoffman and Goodman, 1982), flavoproteins and iron/sulfur proteins (Krieg and Hoffman, 1986), ferredoxins and flavodoxins (Krieg and Hoffman, 1986), tricarboxylic acid cycle (TCA) enzymes (Krieg and Hoffman, 1986) and other enzymes such as fumarate hydratase and malate dehydrogenase (Cole, 1973) and DNA as seen in *Treponema pallidum* (Steiner et al. 1984). Thus there are several theories to justify microaerophilic characteristics of organisms. Stouthamer et al. (1979) give a starting hypothesis for explaining toxicity of atmospheric oxygen pressure to microaerophilic bacteria, this being, "that the respiratory system of these bacteria is organized in such a way that large amounts of toxic oxygen metabolites are formed during respiration at high oxygen pressure and that the activity of enzymes for the decomposition of O_2^- and H_2O_2 is insufficient to prevent damage to the cell. In addition, the physiological properties of microaerophiles might make them more sensitive to the toxic oxygen metabolites".

1.6 NITROGEN FIXING ORGANISMS AND OXYGEN TOLERANCE

1.6.1 Sensitivity of nitrogenase and other components

Among diazotrophs (nitrogen-fixers), organisms belonging to the genus *Azotobacter* are very tolerant of oxygen while fixing nitrogen (Kennedy and Toukdarian, 1987). The enzyme nitrogenase consists of two metalloproteins, both of which are essential for enzyme activity. The larger MoFe protein binds the reducible substrate and the smaller Fe protein interacts with ATP and Mg^{2+} in a hydrolytic reaction (Robson and Postgate, 1980). Both proteins are irreversibly destroyed by oxygen, the latter being more sensitive (Eady and Smith, 1979), and therefore, protection of these proteins is a necessity for nitrogen-fixing cells. The oxygen lability of these proteins involves metal-S centres in each protein which participate in substrate binding or electron transfer. Other oxygen sensitive components of nitrogen-fixing systems are the electron carriers to nitrogenase, the ferredoxins and flavodoxins (Robson and Postgate, 1980), both of which can spontaneously autooxidize resulting in the generation of superoxides (Yates and Jones, 1974). Extreme oxygenation of aerobic nitrogen-fixing cells can result in the generation of superoxides thus increasing the natural oxygen lability of the nitrogen-fixing system (Robson and Postgate, 1980). Although a large proportion of diazotrophs require oxygen as the terminal electron acceptor for energetic pathways, some are facultative anaerobes and a few are strict aerobes (Bergersen, 1984). In terms of efficient nitrogen-fixation, most O_2-tolerant diazotrophs are microaerophilic organisms. Many microbial habitats support microaerobic nitrogen fixation as has been demonstrated for *Campylobacter* spp. (McClung and Patriquin, 1980), *Mycoplana* spp. (Pearson et al. 1982) and *Azospirillum* spp. (Dobereiner and Day, 1974).

1.6.2. Respiratory protection and conformational protection of nitrogenase in response to oxygen stress

Azotobacter species demonstrate a rapid rate of oxygen consumption, faster than solution of oxygen (Kennedy and Toukdarian, 1987). This respiratory protection of nitrogenase maintains nitrogenase in an essentially anoxic environment inside the cells (Kennedy and Toukdarian, 1987). The organism is able to scavenge oxygen by means of an energetically poorly coupled but enhanced respiration (Bergersen, 1984). The rapid switch-on and switch-off of nitrogenase in response to oxygenation and conformational protection of nitrogenase due to association with an FeS protein (Drozd and Postgate, 1970) is an additional protection mechanism *Azotobacter* has evolved for protection of its nitrogenase.

1.6.3 Other mechanisms of oxygen protection of nitrogenase

Several other mechanisms of oxygen protection of nitrogenase are seen in various diazotrophs. Among these are, behavioural and growth adaptations as seen in cyanobacteria which demonstrate clustering (Carpenter and Price, 1976) and azospirilla which congregate to form an oxygen-consuming mass which migrates to a suitable location in an oxygen concentration gradient, and migrates with the oxygen as it is consumed (Barak et al. 1982); morphological adaptations such as heterocyst formation as seen in oxygenic photosynthetic cyanobacteria (Stewart, 1980) so as to physically separate the oxygenic photosynthetic reactions from the nitrogenase; gum formation (Robson and Postgate, 1980); and the well known root nodules of legumes where the concentration of oxygen in the air-spaces of the central tissue is low (Bergersen, 1984)) due to the barrier created by the inner cortex to the inward diffusion of oxygen.

Under excessive oxygenation, the synthesis of nitrogenase is turned off (Robson, 1979) in *Azotobacter*. Oxygen can also inhibit the synthesis of nitrogenase in *Klebsiella pneumoniae* and *Bacillus polymyxa* which therefore behave as anaerobes or microaerophiles (Robson and Postgate, 1980) under nitrogen-fixing conditions.

1.7 REGULATION OF NITROGEN FIXATION

The *nif* genes have been studied the best in *Klebsiella pneumoniae*, although recently, the interest in regulation of *nif* genes in *Azotobacter* spp. has increased. Jacobson et al. (1989) determined the nucleotide sequence of about 17 kb from the *A. vinelandii* chromosome within the major *nif* cluster. The physical organizations of *nif* genes from *K. pneumoniae* and *nif* genes within the major *nif* cluster from *A. vinelandii* have been found to be similar (Jacobson et al. 1989). However, azotobacters are different because they contain more than one type of nitrogenase (Bishop et al. 1980), viz., the well-known molybdenum-based enzyme, the vanadium-based enzyme which is found in both *A. chroococcum* and *A. vinelandii*, when levels of molybdenum fall below 10 nM concentrations (Hales et al. 1986; Robson et al. 1986) and a third nitrogenase in *A. vinelandii*, whose structure is yet to be determined (Chisnell et al. 1988).

In *K. pneumoniae*, *nif* gene expression is controlled by the global nitrogen regulatory elements, NtrA, NtrB and NtrC and it is also subjected to *nif*-specific positive and negative regulation via *nifL/nifA* (Santero et al. 1988). The *ntrC* gene product is a transcriptional activation element, whose activity is modulated by the *ntrB* gene product which is the physiological N-sensory element (Bennett et al. 1988). The *ntrA* gene product is an RNA polymerase sigma factor which provides RNA polymerase

recognition of '*ntr*-dependent' promoter sequences. The 8 *nif* operons in *K. pneumoniae* have promoters recognized by RNA polymerase associated with a sigma factor, σ^{54}, encoded by *ntrA* (also known as *rpoN* or *glnF*) (Santero et al. 1988). Recognition occurs at a consensus sequence with an invariant GG at -24 bp and GC at -12 bp upstream of the site where transcription begins (Gussin et al. 1986).

nif promoters are activated for expression by the binding of the NifA protein, at 100 to 200 base pairs upstream from the start site where the sequence TGT-N_{10}-ACA is important (Santero et al. 1988). The NtrC activator is required for expression of the *nifLA* promoter (Hunt and Magasanik, 1985). Repression in *K. pneumoniae* involves the *nifL* gene product which is made together with the NifA from the *nifLA* operon (Merrick et al. 1982) and acts as a negative effector (Kennedy et al. 1981; Hill et al. 1981; Merrick et al. 1982) in response to the presence of fixed nitrogen or O_2 (Dixon, 1984). On addition of NH_4^+ to cells of *K. pneumoniae* derepressed for nitrogenase, the synthesis of new mRNA rapidly halts or existing *nif* mRNA's become destabilized (Gosink et al. 1990).

The *nifA* gene from *A. vinelandii* has been sequenced and has considerable homology to *nifA* from other organisms (Bennett et al. 1988). Although the *ntrA* gene product is required for *nif* gene expression in *Azotobacter*, the *ntrC* gene product is not required, unlike that in *K. pneumoniae*. A study by Morett et al. (1990) suggested that repression of *nif* transcription could involve an interaction between NifL and NifA resulting in a reduced affinity of NifA for the upstream activating sequence. Both NifL/NifA and NtrB/NtrC are examples of two-component systems of regulatory proteins which respond to environmental changes (Ronson et al. 1987).

1.8 *NTR*A-DEPENDENT GENES

Interestingly, σ^{54}, the product of the *ntrA* gene is required to transcribe a diverse set of genes (Kustu et al. 1989). It is needed for the expression of the gene encoding glutamine synthetase, glnA, in enteric bacteria (Garcia et al. 1977), genes whose products function in the assimilation of nitrogen, for example, genes encoding amino acid transport components and degradative enzymes (Magasanik, 1982), and genes whose products are needed for biological nitrogen fixation (Dixon, 1984). In addition to the genes involved in nitrogen assimilation, σ^{54}-holoenzyme transcribes genes whose products have different physiological roles. These are the i) *dctA* gene of rhizobia which encodes transport of dicarboxylic acids (Ronson et al. 1987), ii) genes on the TOL plasmid of *Pseudomonas putida*, which are involved in the catabolism of toluene and

xylenes (Dixon, 1986), iii) genes encoding certain components of formate-degradative pathway in *E. coli* (Birkmann et al. 1987), iv) genes encoding hydrogenases involved in oxidation of molecular hydrogen in *Alcaligenes eutrophus* (Romermann et al. 1989), v) genes encoding the hook and filament proteins of *Caulobacter* flagella (Ninfa et al. 1989) and, vi) genes encoding pilins in *Pseudomonas aeruginosa* and *Neisseria gonorrhoeae*. (Ishimoto and Lory, 1989).

Control of transcription at σ^{54}-dependent promoters appears to be accomplished primarily by the modulation of the activity state of activator proteins or due to its abundance (Dixon, 1984; Gussin et al. 1986). Each activator allows σ^{54}-holoenzyme to initiate transcription in response to different physiological conditions (Kustu et al. 1989) and the function of the activators, eg. NtrC, themselves is controlled by various mechanisms (Kustu et al. 1989).

1.9 GENE CLONING

There are two main approaches in cloning a gene of interest from the genome of an organism. In the shot-gun cloning method, genomic DNA and vector are cut with the same restriction enzyme, ligated, transformed into a host, and the transformants screened for the gene of interest. In other cloning experiments, selection of a vector-encoded marker following transformation of the host with the recombinant mixture results in the formation of a gene bank or library of the genome being cloned (Ohman, 1988).

1.9.1 Construction of a genomic library

The perfect genomic DNA library would contain DNA sequences representative of an entire genome (Kaiser and Murray, 1985). The library should contain a manageable number of overlapping clones, and should be stable. The cloned fragments should be large enough to contain whole genes and flanking sequences, but at the same time small enough to be mapped by restriction analysis. Overlapping clones facilitate "walking" into adjacent regions of the genome, and the inserted DNA should be easily excised free of vector sequences to be used as a probe to accomplish this. Phage lambda and cosmid vectors have been found to satisfy these requirements, although, plasmid vectors are easier to manipulate. Cloning vectors like pBR322 cannot be used to clone large DNA fragments because of the instability of the recombinant plasmids, and cloning of smaller fragments into this vector would necessitate the screening of a large number of colonies. Secondly, pBR322 has a narrow host range and therefore cannot replicate in bacterial genera like *Pseudomonas* (Ohman, 1988). Phage vectors allow the cloning of large fragments, thus reducing the number of bank members which need to be screened

to find the gene of interest (Ohman, 1988). Cosmid vectors have a larger capacity (~45 kb), whereas phage λ vectors have a restriction of 20-25 kb on the length of insert (Kaiser and Murray, 1985).

1.9.2 Cosmids as cloning vectors

Cosmids are plasmids that contain λ DNA, which includes the *cos* site (single stranded, 5' projections at each end of the double-stranded DNA, which are 12 nucleotides in length and are complementary in sequence, which join to form the *cos* site). The phage λ can be "tricked" into packaging a plasmid with the *cos* site and having a total length similar to that of the λ genome (40 to 50 kb in size). In addition to the above mentioned advantages of cosmids as cloning vectors, packaging of cosmid clones into protective phage coats permits the efficient transduction of large recombinant plasmids into *E. coli* (Ohman, 1988). Commercially available packaging extracts which contain phage coat particles that self-assemble to package *cos*-containing recombinant plasmids that total 40 to 50 kb in size are used instead of directly transforming competent *E. coli* with the recombinant plasmid (Ohman, 1988). This is because there is a natural barrier against large recombinant molecules in transformation when the plasmid molecules are large (30 to 35 kb) (Ohman, 1988).

Often genes from other organisms are not expressed in *E. coli* because of differences in transcription/translation systems or because of the lack of a biochemical pathway essential for expression of the particular phenotype (Ohman, 1988). Under these circumstances it is advantageous to use a broad-host range cloning vector which replicates in *E. coli* and many Gram negative species. Examples of such vectors are pLAFR1 (Ohman, 1988), and pLAFR3 (Staskawicz et al. 1987). An additional advantage of the vectors mentioned is that they are low copy number (about 4 copies per chromosome) so that expression of a gene mimics expression from the chromosome more closely than if it were replicating at a high copy number (Ohman, 1988).

1.9.3 Identification of recombinant clones

There are several methods to identify bacterial colonies that contain recombinant plasmids. These are by restriction analysis of small-scale preparations of plasmid DNA, α-complementation, insertional inactivation of an antibiotic resistance gene, screening by hybridization (Sambrook et al. 1989), or enzymatic or immunochemical detection of a clone synthesizing the gene product of the desired gene (Ohman, 1988).

1.10 TRANSPOSON MUTAGENESIS

Recently, transposable elements have been increasingly exploited as a tool for mutational analysis and *in vivo* genetic engineering in microorganisms (Berg et al.1989). Their major uses have been in the analysis of transcriptional and translational regulation and protein localization, insertion mutagenesis and the introduction of linked selectable markers, the introduction of engineered genes into microorganisms and in DNA sequencing (Berg et al. 1989). Among these, Tn5 has been used successfully in the genetic analysis of several bacterial species (deBruijn and Lupski, 1984), particularly Gram-negative organisms.

Transposon mutagenesis strategies can be either direct or indirect. In the direct method, the transposon is introduced into the bacterial species of interest, whereas in indirect mutagenesis, the transposon is introduced in the most convenient host containing the cloned DNA fragment and the mutated clone is then returned to the organism of interest (Berg et al. 1989). This indirect procedure has been widely used in the genetic analysis of *Rhizobium* and *Agrobacterium* species (Ruvkun and Ausubel, 1981).

Several Tn5 derivatives have been constructed in vitro, including Tn5-Lux (Boivin et al. 1988), which carries the genes for bioluminescence, and Tn5*tac*1 which generates conditional phenotypes in *E. coli* by regulating transcription of target sequences adjacent to the site of transposon insertion (Chow and Berg, 1988). Tn5 and its derivatives are very useful tools for studying the genetic systems of several organisms because it allows the rapid identification of the location, approximate boundaries and transcriptional organization of cloned genes and operons (deBruijn and Lupski, 1984).

1.11 *AZOTOBACTER CHROOCOCCUM* STRAIN 184

1.11.1 Characteristics of *Azotobacter*

Azotobacters are Gram negative, large ovoid cells, capable of fixing nitrogen, and are widely distributed in soil, water (Jensen, 1954) and the plant rhizosphere (Tchan and New, 1984). They are generally found in soils of slightly acid to alkaline pH. They form cysts and are motile by peritrichous flagella or are non motile (Tchan and New, 1984). The organisms are obligate aerobes but can grow under reduced oxygen tensions. Some strains produce water-soluble or water-insoluble pigments. Different species and strains have various abilities to synthesize alginates, poly-β-hydroxybutyrate and plant hormones (Kennedy and Toukdarian, 1987). They are catalase positive and they grow in a pH range of 4.8-8.5 in the presence of combined nitrogen. The optimum pH for

growth under nitrogen-fixing conditions is 7.0-7.5. Azotobacters are the first nitrogen-fixing organisms found to contain more than one type of nitrogenase enzyme. Both *A. chroococcum* and *A. vinelandii* have the molybdenum-nitrogenase, and a second vanadium-nitrogenase when the level of molybdenum falls below 10 nM (Hales et al. 1986; Robson et al. 1986). In addition, *A. vinelandii* synthesizes a third nitrogenase (Jacobson et al. 1986). The mol% G+C of the DNA is 63.2 to 67.5 (Tchan and New, 1984). The type species is *A. chroococcum* Beijerinck 1901, 567.

1.11.2 Characteristics of *A. chroococcum*

This is the most common *Azotobacter* species in soil with pH above 6, and from fresh waters (Thompson and Skerman, 1979). The organism has peritrichous flagella, grows in a pH range of 6.5-10 under nitrogen-fixing conditions and a temperature range of 14-32°C, although some strains show optimum growth at 37°C (Tchan and New, 1984). On nitrogen-free medium, it produces a nondiffusible brown-black pigment, which is medium-dependent. Some strains produce a diffusible brown-black pigment in the presence of benzoate (Tchan and New, 1984). The organism demonstrates white sectors in normally pigmented colonies due to mutation-like changes, which appear to be unstable (Lipman and McLees, 1940). Several strains of *A. chroococcum* harbour plasmids, whose properties have not been conclusively identified (Robson et al. 1984). The presence of plasmids is thought to be responsible for the decreased efficiency with which this species accepts DNA either by transformation or conjugation (Robson et al. 1984). The type strain is *A. chroococcum* ATCC 9043.

1.11.3 Characteristics of strain 184

Strain 184 was identified as an *A. chroococcum* species based on characteristics described above (Page, 1986). However the organism, which was isolated from Alberta soils is a capsule-negative strain and produces black colonies on normal plating media. It has an absolute dependence on Na^+ for growth, the optimum concentration being 0.8 mM (Page, 1986). Growth is limited at 0.2 to 0.35 mM Na^+. The Na^+-requirement is demonstrated under all growth conditions, under nitrogen-fixing and in the presence of fixed nitrogen sources and throughout the pH range of growth (6.2 to 8.0) (Page, 1986).

The organism also demonstrates an inability to grow in iron-limited media (Page, 1987) and excretes citric acid and succinic acid (Page, 1987) in the presence and absence of iron. It produces a hydroxamate-type of siderophore (Page, 1987), the production of which is optimal at 0.75 μM ferric citrate and is repressed by concentrations > 5 μM.

From the growing physiological data and recent 16S rRNA sequencing data (E.R.B. Moore, G.E. Fox, and P. Jurtshuk, Jr. Abstract # 251, p. 196: Abstracts of the 90th Annual Meeting of the American Society for Microbiology, Anaheim, California,

13-17 May, 1990) the dissimilarity between this organism and *A. chroococcum* species has become apparent. Based on these studies, it is being proposed that the organism be renamed *Azotobacter salinestris* and strain 184 be considered the type strain (Page and Shivprasad, submitted for publication).

1.12 THESIS OBJECTIVES

Preliminary studies demonstrated that strain 184 exhibits characteristics quite different from the type strain ATCC 9043. The object of my thesis was to further characterize strain 184 including studying the oxygen requirements and oxygen tolerance of the organism. This study reports the identification of the microaerophilic characteristics of the organism, including the lack of the enzyme catalase; the isolation and identification of catechol (which was polymerized to a melanin) from culture supernatants of nitrogen-fixing cells; the construction of a genomic library of the organism in the broad host range cosmid cloning vector, pLAFR3; and the subsequent isolation of a melanin-producing clone. The studies indicated that the organism shares characteristics with microaerophilic organisms and produces catechol, the formation of which was repressed by NH_4^+, and its regulatory characteristics resembled that of the *nif* genes. The polyphenol oxidase gene of the organism was cloned in *E. coli*, and Tn5 mutagenesis was carried out as a preliminary step to study the regulatory mechanisms involved in melanin formation. A role for catechol and/or melanin formation in aeroadaptation is proposed.

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CHAPTER 2

Sodium-dependent *Azotobacter chroococcum* strains are Aeroadaptive, Microaerophilic, Nitrogen-Fixing Bacteria.

2.1 INTRODUCTION

Members of the genus *Azotobacter* are known to be free-living, nitrogen-fixing, obligate aerobes (Becking, 1981). These bacteria possess the highest cellular respiratory rate of any known organism as was first described by Lineweaver (1933). Their rapid consumption of oxygen allows them to grow well and to fix nitrogen under extreme aeration conditions, for example, 40 to 50% O_2 with intense vortex mixing (Hine and Lees, 1976). However, the efficiency of nitrogen fixation is higher at low oxygen tensions (Parker, 1954). Under conditions of high aeration, respiration is adjusted to achieve zero dissolved oxygen in the cell, affording a type of respiratory protection to the oxygen-labile nitrogenase (Dalton and Postgate, 1969; Drozd and Postgate, 1970). When respiratory protection is inadequate, synthesis of nitrogenase de novo is repressed (Robson, 1979) and the existing nitrogenase is stabilized by conformational protection (Robson and Postgate, 1980). These cells also typically have a very active catalase and superoxide dismutase (SOD), enzymes to protect the cells from toxic oxygen products formed as a result of this high metabolic and respiratory activity (Jurtshuk et al. 1984; Dingler and Oelze, 1987).

Recently, *Azotobacter chroococcum*, absolutely dependent on Na^+ for growth, has been isolated from soils of Alberta, Canada (Page, 1986). By using this strain 184, a capsule-negative variety, it has been shown that Li^+ and Mg^{2+} can substitute for part of theNa^+-requirement, whereas K^+ and Rb^+ are ineffective and growth-inhibitory substances (Page, 1986). These ion requirements are quite like those of marine bacteria (Reichelt and Baumann, 1974), an intriguing observation in light of the soil origin of these bacteria.

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As would be expected, members of the family *Azotobacteraceae* have a requirement for iron for optimal growth, respiration, oxygen protection, and numerous other redox reactions carried out by the cell (Page and Huyer, 1984; Page and Collinson, 1987). These bacteria are capable of iron-starved growth, which is accompanied by the production of specific chelators of iron, the siderophores (Neilands, 1981). However, Na^+-dependent *A. chroococcum* is incapable of iron-starved growth (Page, 1987). These cells require a small amount of iron, either from the medium or as a carry-over of intracellular iron in the inoculum, for growth and siderophore production under iron-limited conditions (Page, 1987). The production of a hydroxamate-type of siderophore, citric acid and succinic acid by this organism has been reported earlier. Citric acid is thought to play a role in low-affinity iron uptake in this organism (Page, 1987).

The present study was primarily initiated to determine whether the iron-dependent growth phenotype of Na^+-dependent *A. chroococcum* was related to a failure to protect the cells from toxic oxygen products under conditions of limited iron. While examining this possibility, we observed that even iron-sufficient Na^+-dependent *A. chroococcum* routinely gave a negative reaction in the H_2O_2 spot test for catalase activity and that these cells appeared to share a number of growth characteristics with microaerophilic bacteria. Further characterization of this organism was also carried out in order to determine its oxygen and carbon source preferences under various oxygen tensions.

Because of the importance of iron accumulation in these bacteria, this study further examines the hydroxamate siderophore and the iron-uptake system of strain 184. Preliminary characterization of the hydroxamate-siderophore was carried out in this study. In addition, a plasmid was discovered in strain 184 and it was characterized in this study. Although the presence of indigenous plasmids has been well documented in *A. chroococcum* (Robson et al. 1984; Saitia et al. 1989), a defined role for these plasmids has remained elusive.

2.2 MATERIALS AND METHODS

2.2.1 Bacterial strains and growth conditions

The *A. chroococcum* strain used in this study was the Na^+-dependent, capsule-negative strain 184. The *A. chroococcum* type strain ATCC 9043, *Azotobacter vinelandii* OP strain UW (Page and Huyer, 1984) and *Azomonas macrocytogenes* NCIB 8702 were also used.

These strains were grown on the Burk's medium described previously (Page, 1987). Nitrogen-free medium did not contain the normal 1.1 g of ammonium acetate liter^{-1}, and the iron content of the medium was varied by the addition or deletion of ferric citrate (Page, 1987). The carbon source used was glucose (1%, w/v) unless otherwise stated. For studies on the hydroxamate, glassware was first washed with 4 M HCl and then with 50 mM EDTA, pH 7, prior to use, to minimize contamination by iron. Media were always prepared using water from the Milli-Q water purification system (Millipore Corp., Bedford, MA). The medium was inoculated and incubated at 30°C with gyratory shaking at 225 rpm, unless otherwise stated, in a New Brunswick model G-76 water bath shaker as described previously (Page, 1987). Protein was estimated by the method of Lowry et al. (1951) or Bradford (1976). For the Bradford assay, the cell pellet was resuspended in 1N NaOH and the suspension incubated at 30°C upto a maximum of 2 hours. Aliquots were then analyzed for protein content.

More vigorous aeration, by shaking at 250 rpm and decreasing the medium-to-flask volume ratio from 40 to 10%, was used to cause oxygen stress. Burk's medium (10 mL) containing 0.05% agar in 16-mm diameter tubes was used for static culture. Alternatively, the NFb medium of Okon et al. (1976) was used which contained, per litre: K_2HPO_4, 6 g; KH_2PO_4, 4 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; NaCl, 0.1 g; $CaCl_2$, 0.02 g; $FeCl_3$, 0.01 g; $Na_2MoO_4 \cdot 2H_2O$, 0.002 g. The pH was adjusted to 6.8 with NaOH. The medium was made semisolid by adding 0.05% agar and the carbon source was 0.5% sodium pyruvate unless otherwise stated. Protection of petri plate cultures from atmospheric oxygen was achieved by incubation in a 5%O_2-10% CO_2 environment created by the gas-generating kit intended for *Campylobacter* culture (BR60; Oxoid Ltd.).

2.2.2 Acetylene reduction under varying head space oxygen levels

Strain 184 was grown in serum bottles (48 mL/160 mL total volume) which were sealed with serum stoppers after inoculation. The bottles were then flushed with air for one minute and incubated at 30°C in a gyratory water bath shaker at 250 rpm. At particular time intervals they were analyzed for nitrogen-fixing ability. For this, the bottles were flushed with helium for 2 minutes (to remove nitrogen which would otherwise compete with acetylene). Levels of oxygen in the head space were varied by replacing helium with oxygen (eg. for 5% oxygen in the head space, 16.8 mL helium was removed and replaced with the same amount of oxygen). These values are expressed as the initial head space concentration of oxygen since in the 1.5 hour incubation period during the assay, the level of oxygen would decrease. 10% of the head space was also replaced with acetylene. The bottles were then incubated at 250 rpm in the shaker for 1.5 hours after which the gas phase was analyzed for ethylene by injecting

0.5 mL of the gas phase into a Hewlett-Packard gas chromatograph (model 5890A) fitted with a Poropak R column and a flame ionization detector. The carrier gas was helium (~ 51 cm/sec). The column temperature and injector temperature were maintained at 60°C. The specific activity of nitrogenase was expressed as micromoles of ethylene produced milligram of cell protein^{-1} hour^{-1}. There was variation in results between replicate experiments. Between duplicates in the same experiment there was variability up to 20%. Protein estimations were carried out by the method of Bradford (1976).

2.2.3 Growth and nitrogen-fixation at different aeration rates

To study growth and nitrogen-fixing ability at different aeration rates, the cultures (15 mL in 50-mL Erlenmeyer flask) were incubated at 30°C at different shaking rates in a gyratory water-bath shaker, viz., 250 rpm, 200 rpm and 150 rpm, or static. After the required period of incubation, nitrogen-fixing ability was determined by transferring 3 mL of cell suspension to a 10-mL Erlenmeyer flask which was sealed with a serum stopper and had 10% of the atmosphere replaced by acetylene. The flasks were then incubated for 1.5 hours at the same aeration condition as that used for growth unless otherwise stated. The gas phase was then analyzed for ethylene by the procedure described above.

To study the carbon source requirement under aerobic and microaerobic conditions, the organism was grown with various organic acids and carbohydrates. Organic acids were used as their sodium salts, and the Na^+ levels adjusted in all the flasks with NaCl. Under aerobic conditions, the carbon source was used at 30 mM concentration, whereas under microaerobic conditions, at 10 mM levels. The medium used was Burk's medium with 0.18% $CaCO_3$ and 10 µM ferric citrate. To establish aerobic conditions, the organism was grown in flasks (15 mL medium per 50-mL Erlenmeyer flask) and for microaerobic conditions, it was grown in test tubes (5 mL medium with 0.05% agar per 13 mm test tube). Washed cultures were used to inoculate the media. After 22 hours incubation, 3 mL cell suspension was analyzed from the aerobically grown cells by the procedure described above. For the microaerobically-grown cells, the test tubes were stoppered with serum stoppers and 10% of the atmosphere was replaced with acetylene. The aerobically-grown cells were incubated at 250 rpm, whereas, the microaerobically-grown cells were incubated under static conditions during the acetylene reduction assay. 0.5 mL of the gas phase was analyzed as described above.

2.2.4 Variations in plating media

Plate count assays were conducted after dilution of overnight cultures in Burk's buffer (Burk's medium lacking carbon and nitrogen) containing 50 µg of NaCl mL^{-1}.

The plating medium consisted of Burk's medium containing 18 µM iron, 1% glucose, and 1.1 g of ammonium acetate $litre^{-1}$, solidified with 1.5% agar. The medium was treated in the following different ways: (i) autoclaved for 17 min at 1.1 kg cm^{-2} at 121°C; or (ii) autoclaved and exposed overnight to a 15-W cool-white fluorescent light, mounted 30 cm from the agar surface; or (iii) autoclaved with the addition of 0.2% activated charcoal (100 to 400 mesh; Sigma Chemical Co.); or iv) autoclaved but solidified with 8 g of gellan gum (K9A40; Kelco) $litre^{-1}$ and 0.9 g of $MgCl_2 \cdot 6H_2O$ $litre^{-1}$. The plating medium also was dissolved by heating in a microwave oven and sterilized by forcing the hot fluid through a 0.45 µm-pore-size Millipore filter. Catalase (88 U mL^{-1}; Sigma) also was added to some plates after they were autoclaved. All media were protected from the light after they were poured into plastic petri dishes, except as noted above.

2.2.5 Enzyme activity measurements

Cell extracts were prepared as described by Page and von Tigerström (1982). Protein assays were performed as described previously (Page and Huyer, 1984). All strains were harvested after 24 hours of incubation except strain 9043 which was harvested after 48 hours.

Catalase activity was initially observed by adding 5% H_2O_2 to fresh cultures grown on solid medium. The use of up to 20% H_2O_2 did not alter the results with this spot test. Catalase activity was assayed by following H_2O_2 degradation coupled to a colorimetric assay involving the reduction of dichromate to chromic acid in the presence of acetic acid and residual H_2O_2 (Sinha, 1972). The assay was scaled down to 5 mL, and samples were taken at 1, 3, and 5 minutes. Activities were calculated from 0- to 3-minutes or 3-to 5- minute periods, when H_2O_2 was decreasing at a linear rate. One unit was defined as 1.0 µmol of H_2O_2 degraded $minute^{-1}$.

o-Dianisidine peroxidase (ODPX) was assayed by a method based on that of Guidotti et al. (1961). The reaction mixture (2.9 mL) contained 10 mg of o-dianisidine mL^{-1} and 0.265% H_2O_2 in 0.06 M potassium phosphate buffer (pH 7.0) mixed with 0.1 mL of cell extract. After incubation at 37°C for 1 hour, the pH was adjusted to <2 with HCl to stabilize the colour. One unit of activity was the change in the A_{400} $hour^{-1}$.

p-Phenylene-diamine peroxidase (PDPX) was assayed spectrophotometrically following the oxidation of p-phenylenediamine at 485 nm (Lück, 1963). Units of activity were calculated by using a molar extinction coefficient of 1.545×10^3 $M^{-1}cm^{-1}$ (Allgood and Perry, 1985) and defined as 1.0 µmol of p-phenylenediamine oxidized $minute^{-1}$.

SOD was assayed according to the method of Beauchamp and Fridovich (1971). The assay was based on the ability of SOD to inhibit the reduction of Nitroblue tetrazolium by $O_2^{\cdot-}$. One unit was defined as the amount of SOD causing half the

maximum inhibition of Nitroblue tetrazolium reduction (Beauchamp and Fridovich, 1971). The relative mobility and iron requirement of SOD from *Azotobacter* spp. was determined after polyacrylamide gel electrophoresis as described by Jurtshuk et al. (1984).

2.2.6 Purification of siderophore

The organism was grown in Burk's medium containing different iron minerals, viz. glauconite ($K_2(Mg,Fe)_2Al_6(Si_4O_{10})_3(OH)_{12}$), olivine ($(Fe,Mg)_2SiO_4$) or Fe_3O_4 and the carbon sources were varied to determine the optimum conditions for hydroxamate production. After 5 days growth the culture was centrifuged at 10,400 x g for 10 minutes to collect the supernatant. The supernatant was concentrated by rotary evaporation using a Buchi Rotavapor-R at 30 to 34°C. The procedure used for purification of the siderophore was that of Byers et al. (1967). A preliminary ethyl acetate extraction was carried out to remove the catechol which was present. The aqueous phase was then evaporated to dryness, redissolved in Milli-Q water and 0.3% (w/v) charcoal (14/600 mesh; Sigma) (which had been cleaned before use by successive washes with ethyl alcohol, acetone, ethyl alcohol:acetone:7% ammonia (50:25:10) was then added to adsorb the hydroxamate. The adsorbed hydroxamate was eluted with ethyl alcohol:acetone:7% ammonia (50:25:10) and the eluate contained about 80% of the hydroxamate which was present in the original culture supernatant. The eluate was again dried down and dissolved in water. The sample was then passed down a Sephadex LH-20 column which was equilibrated with water and fractions then eluted using water as eluant. The intense yellow eluate, which was hydroxamate-positive, in the early fractions was then extracted into ether. The ether was evaporated and the compound redissolved in chloroform. The solubility of the compound in various solvents was determined. TLC analysis of the partially-purified compound was carried out using ethanol-acetone-7% ammonia as solvent. Iron-binding compounds were detected using the spray reagent ferric chloride (0.1 M $FeCl_3 \cdot 6H_2O$ in 0.1M HCl) and 2,2'-dipyridyl-ferric chloride (Krebs et al. 1969). Amino acids were identified using ninhydrin (0.2% in acetone) as spray reagent (Krebs et al. 1969). $^{55}Fe^{3+}$-uptake assays were carried out by the procedure of Knosp et al. (1984). In this procedure citrate is used as iron chelator during the assay. Since citrate mediates iron uptake in strain 184, 6 µM EDDHA (Ethylene diamine di-(o-hydroxyphenyl acetic acid) replaced citrate in the assay. Quantitative estimation of hydroxamate was carried out using the procedures of Csáky (1948) with hydroxylamine hydrochloride as standard or that of Arnold and Viswanatha (1983) with desferal as standard. This latter assay is based on the competition for ferric ions between a bis(mercaptoacetato-S,O)hydroxoiron(III) complex and the hydroxamate-siderophore.

2.2.7 Plasmid isolation

Plasmid isolation from strain 184 was carried out essentially by the procedure of Robson et al. (1984) with minor modifications. From 150 mL, the cells were collected by centrifugation at 10,400 x g for 10 minutes at 4°C. The pellet was resuspended in PEM (5 mM K_2HPO_4, 0.1 mM EDTA, 0.5 mM $MgCl_2$, pH 8) and the cells washed once. The pellet was then resuspended in 5 mL TES (10 mM Tris, 50 mM EDTA, pH 8) and 95 mL freshly prepared alkaline-SDS (SDS, 35 mM adjusted to pH 12.6 with NaOH) was added and mixed rapidly with the suspension. After incubation for 20 minutes at 34°C, the denatured DNA, protein and SDS was precipitated by incubating the suspension at 0°C for 20 minutes after adding 17 mL of a solution containing 3.5 M NaCl, 0.7 M Tris, pH 8. After centrifugation at 4000 x g for 10 minutes at 4°C, the supernatant was extracted once with phenol, twice with phenol:chloroform (1:1) and once with chloroform. The DNA was then precipitated with ACE (Na acetate, 0.2 M in 90% v/v ethanol). After incubation at -70°C for 2 hours the DNA was collected by centrifugation at 10,000 x g for 30 minutes. at 4°C. The pellet was redissolved in TES and RNAase digestion (10 µg/mL) carried out at 37°C for 30 minutes.

The plasmid was analyzed by gel electrophoresis by the procedure described in Sambrook et al. (1989). Restriction enzyme digestions were carried out by standard procedures (Sambrook et al. 1989). The size of the plasmid was estimated by comparing the size of the fragments with lambda DNA cut with *Hind*III and BstEII which gives rise to fragments of known sizes. In another approach, the multiple plasmid-containing *E. coli* V517 (Macrina et al. 1978) was used to estimate the size of the plasmid.

2.3 RESULTS

2.3.1 Studies on the hydroxamate siderophore produced by strain 184

In order to determine the optimal conditions for hydroxamate production, the organism was grown with various iron minerals viz., glauconite, olivine and Fe_3O_4. Among these, olivine and Fe_3O_4 supported the highest hydroxamate production under both nitrogen-fixing and $+NH_4^+$ conditions (data not shown). Glauconite supported good growth, but low levels of hydroxamate were produced. Thus Fe_3O_4, at 25 mg/mL was used for further studies. Among the carbon sources, mannitol and sucrose supported the maximum hydroxamate production which was achieved in 5 days (Table 2.1). It was also observed that cells grown for this period of time turned brown,

Table 2.1 Effect of carbon source on hydroxamate production[a]

	Hydroxamate				
Nitrogen source	D-Glucose	Maltose	Melibiose	Mannitol	Sucrose
N_2	0.13	0.15	0.18	0.30	0.26
NH_4^+(14 mM)[c]	0.13	0.12	0.15	0.26	0.22

a All values are means of at least two duplicate determinations. The carbon sources were provided at 2% (w/v) concentration. Cultures (100 mL per 500 mL Erlenmeyer flask) were harvested after 5 days incubation. Hydroxamate was estimated by the method of Arnold and Viswanatha (1983). Protein was estimated by the method of Lowry et al. (1951).

b Expressed as micromoles desferal equivalents (milligram of protein)$^{-1}$.

c NH_4^+ was added as ammonium acetate.

probably due to the production of the black pigment, melanin. This effect was seen particularly with nitrogen-fixing cells (see Chapter 3). Thus for maximum hydroxamate production, the cells were grown in Burk's medium supplemented with NaCl (0.85 mM), sucrose (2%) and Fe_3O_4 (25 mg/mL), for 5 days, after which the culture supernatant was used for the extraction of hydroxamate. Preliminary characterization of the hydroxamate showed that the compound was hydrophilic and polar. It was soluble in acetone, benzene, diethyl ether, ethanol and methanol. It was insoluble in hexane and phenol:chloroform (1:1). Analysis by thin layer chromatography using silica gel-G plates identified a ninyhdrin-reactive band in hydrolyzed samples. The identification of this amino acid was not carried out. Several schemes to further purify the hydroxamate were carried out but were unsuccessful.

$^{55}Fe^{3+}$-uptake assays where concentrated culture supernatant (having ~ 4 nmoles desferal equivalents in the uptake assay solution) were used demonstrated that the hydroxamate did indeed mediate uptake (data not shown). Among the other iron chelators, Nitrilotriacetic acid (NTA) also mediated uptake in the absence of culture supernatant. Desferal did not mediate iron assimilation in this organism. Quantitative studies to express the amount of cell-associated $^{55}Fe^{3+}$ per cell could not be established due to the inability to obtain reliable viable counts by the plate count method. The organism appeared not to be able to survive under the normal plating conditions.

2.3.2 Oxygen toxicity on solid media

Oxygen toxicity to bacterial cells has been observed to be more pronounced at low cell densities and when plated on solid media (Hoffman et al. 1979; Padgett et al. 1982; Hoffman et al. 1983). Also, H_2O_2 is generated spontaneously in aerobic, illuminated culture media in sufficient quantity to be growth inhibitory (Padgett et al. 1982; Hoffman et al. 1983). In other studies with strain 184, it was noted that cell suspensions that were metabolically active could not be enumerated by the plate count assay (unpublished data). Platings of strain 184 usually did not yield counts proportional to dilution, especially when the number of expected cells per plate was less than several hundred. The recovery of iron-sufficient cells on normal, autoclaved agar medium (denoted 'control' treatment) was <20% of the total count and was approximately 2% when the cells were from iron-starved cultures (Table 2.2). The recovery of iron-starved (0 μM Fe^{3+}) and iron-limited (0.35 μM Fe^{3+}) cells was even less when the autoclaved agar medium had been illuminated overnight prior to plating. The addition of charcoal, which is known to prevent photochemical oxidation reactions and to absorb the reaction products (Hoffman et al. 1983), increased the recovery of strain 184 (Table 2.2). Similarly, filter sterilization, which decreases the tendency of media to undergo photochemical generation

Table 2.2 Comparison of the recovery of strain 184 on different plating media

Medium treatment[a]	% Recovery[b] (n)		
	0 μM Fe^{3+}	0.35 μM Fe^{3+}	10 μM Fe^{3+}
Control	1.94 ± 0.68 (5)	5.41± 4.28 (4)	15.9 ± 6.4 (5)
Light	0.38 ± 0.19 (2)	0.47 ± 0.08 (2)	21.0 ± 13.8(2)
Filtered	35.6 ± 6.10 (5)	35.0 ± 5.20 (4)	44.4 ± 6.3 (5)
Charcoal	23.8 ± 5.90 (4)	36.8 ± 4.20 (3)	39.0 ± 5.6 (4)
Gellan gum	15.1 ± 5.40 (4)	13.6 ± 5.80 (4)	13.9 ± 5.9 (4)

[a] All media were prepared from Burk's medium containing 18 μM iron and ammonium acetate as the nitrogen source. Treatments of the plating media are described in Materials and Methods.

[b] Recovery relative to the total count of each culture as determined with a Petroff-Hausser counting chamber. The cultures containing the iron contents noted were incubated overnight before the viable count assay. Values are expressed as mean ± standard error of the mean.

of toxic oxygen products (Hoffman et al. 1983), improved the recovery of strain 184 (Table 2.2). The use of the nonagar gelling agent, gellan gum, also resulted in higher viable counts with iron-starved and iron-limited cells, but gellan gum appeared to suppress the growth of iron-sufficient cells (Table 2.2). The recovery of viable cells was not improved by incubating the plates in 5% O_2 or by including catalase in the plating medium (data not shown).

2.3.3 Sensitivities to H_2O_2, paraquat and metronidazole

Because of the sensitivity of strain 184 to toxic oxygen products generated in laboratory media, its sensitivity to hydrogen peroxide and paraquat (which generates toxic oxygen products) were studied. Small amounts of H_2O_2 were inhibitory to the growth of strain 184 (Fig. 2.1). Approximately 50% inhibition of growth was caused by 0.04 mM H_2O_2. This effect was obvious with iron-sufficient cells, which indicated a normal inability to deal with H_2O_2. In comparison, strain 9043 was unaffected by 0.1 mM H_2O_2 and was inhibited 50% by approximately 0.3 mM H_2O_2 (Fig. 2.1).

The addition of paraquat (methyl viologen) to culture media and the ensuing generation of $O_2^{\cdot}$ and $\cdot OH$ is toxic to microorganisms, depending on their abilities to protect themselves from these toxic oxygen products (Carr et al. 1986). Strains 184 and 9043 proved to be very sensitive to paraquat and were inhibited 90% by approximately 0.25 and 0.1 μM paraquat, respectively (Fig. 2.2). Cells grown in iron-sufficient cultures were considerably more sensitive to paraquat than those grown in iron-limited cultures. There was no significant increase in catalase or peroxidase activity in these strains in response to paraquat (data not shown).

Anaerobic organisms and some microaerophiles are known to be sensitive to metronidazole (Krieg and Hoffman, 1986). A study of metronidazole sensitivity demonstrated that *A. chroococcum* strains 9043 and 184 and *A. vinelandii* strain UW were all sensitive to metronidazole under nitrogen-fixing conditions (Fig. 2.3). However, the extent of sensitivity varied among the organisms. *A. chroococcum* strain 9043 showed the highest sensitivity, its growth being inhibited by 59% by 10 μg/mL metronidazole. At the same concentration, strain 184 was inhibited only 15% and *A. vinelandii* UW by 11%. At higher concentrations of metronidazole, strain 184 showed a higher sensitivity than *A. vinelandii* UW (Fig. 2.3).

2.3.4 Catalase and peroxidase activities.

When the H_2O_2 spot test for catalase activity was performed on a variety of *A. chroococcum* strains, the majority were clearly catalase positive (data not shown; Page et al. 1988). The Na^+-dependent strains, however, often gave negative results. Examination of cell extracts, however, revealed that catalase activity was present in all of

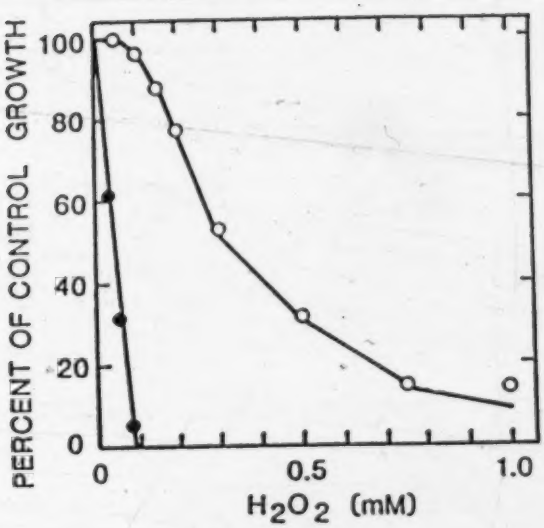

Fig. 2.1. Comparison of the sensitivities of strains 184 (•) and ATCC 9043 (o) to H_2O_2. Growth (micrograms of protein per milliliter measured after 24 hours of incubation with shaking at 225 rpm in Burk's medium containing 10 μM Fe^{3+} and ammonium acetate) is shown as a percentage of growth of untreated controls.

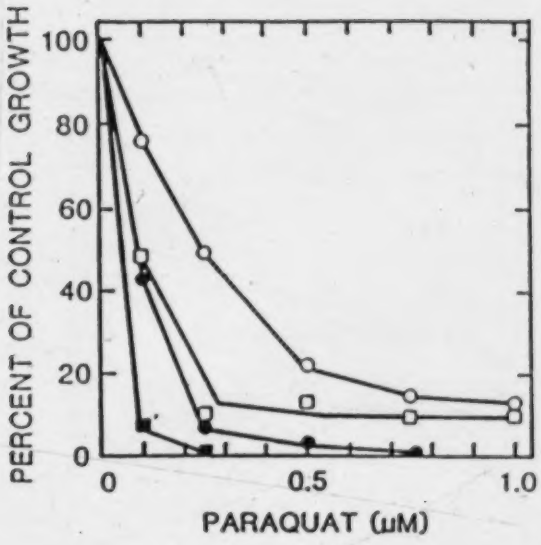

Fig. 2.2. Comparison of the sensitivities of strain 184 (○,●) and ATCC 9043 (□,■) to paraquat. Growth was calculated as in Fig. 2.1. The medium contained either 0.35 (○,□) or 10 (●,■) μM Fe^{3+}.

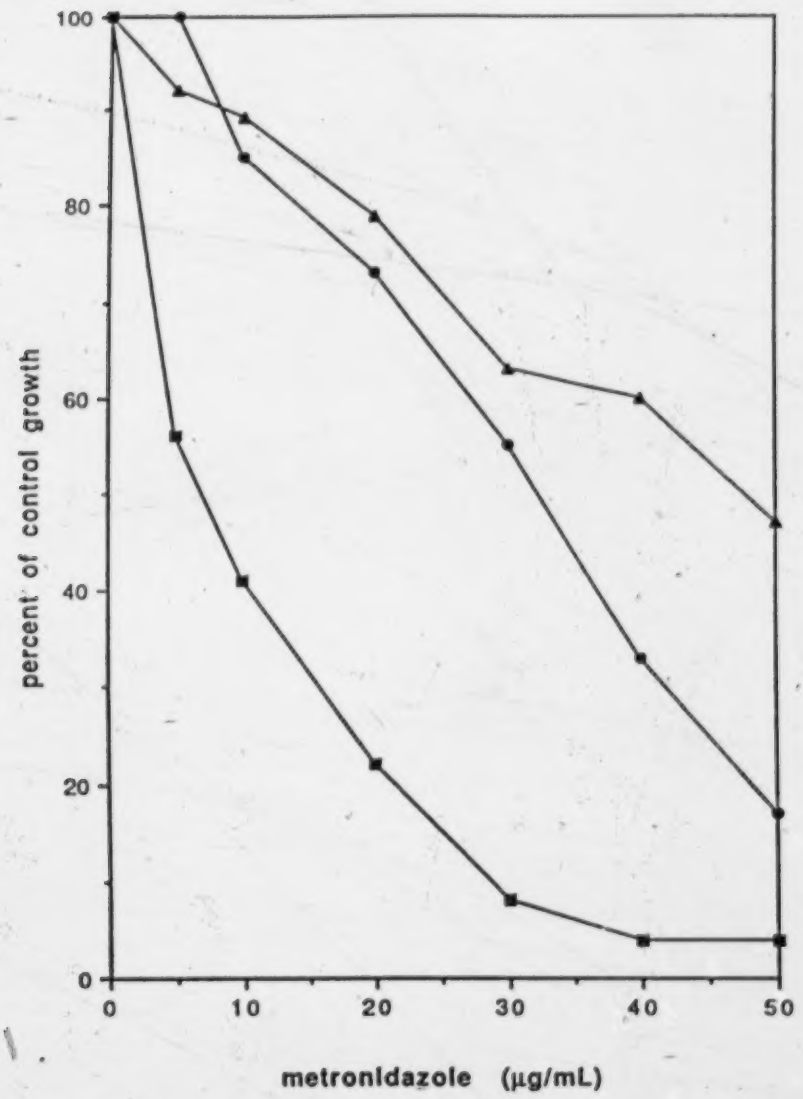

Fig. 2.3. Comparison of the sensitivities of strain 184 (●), ATCC 9043 (■) and *A. vinelandii* UW (▲) to metronidazole. Growth was calculated as in Fig. 2.1. The organisms were grown under nitrogen-fixing conditions in medium containing sucrose (2% w/v) as the carbon source. Growth was estimated by the method of Bradford (1976).

the strains (Page et al. 1988). Strain 184 had a catalase activity that was 60- to 500-fold less than those of Na^+-independent strains. This activity in strain 184 appeared to be real but was at the lowest limit of detection in the assay (Table 2.3). For example, detection of catalase activity in strain 184 required the use of ≥ 500 µL of cell extract containing ≥3 mg of protein mL^{-1}. By comparison, catalase activity was clearly present in strain 9043 when 15 µL of cell extract containing ~0.5 mg of protein mL^{-1} was used.

Strain 184 was examined further to determine whether a peroxidase activity was present that would supplement the low catalase activity. The ODPX activity in strain 184 was not reliably greater than that found in the Na^+-independent strain 9043 (Table 2.3). PDPX activity was also present in strain 184 (Table 2.3) and was present in only the Na^+-dependent strains (Page et al. 1988). Strain 9043 did not give a negative result because of interference with this assay. For example, a 1:1 (vol/vol) mixture of cell extracts from strain 184 and 253 (a Na^+-independent strain) decreased the PDPX activity in proportion to the dilution (data not shown).

2.3.5 Iron-dependence of catalase and peroxidase activities

Cell extracts from strain 184 incubated overnight under conditions of iron-starvation (0 µM Fe^{3+}) contained no detectable catalase and minimal peroxidase activities (Table 2.3). SOD, however, was present and active (Table 2.3). With limiting (1 µM) and excess (10 µM) Fe^{3+}, the activities of catalase and peroxidase increased with increasing iron availability (Table 2.3).

SOD activity in strain 184 was not affected by Fe^{3+} availability (Table 2.3). When the cell extract was subjected to polyacrylamide gel electrophoresis and assayed for SOD activity, the SOD had a relative mobility of 0.6 (data not shown), similar to that reported for other *A. chroococcum* strains (Jurtshuk et al. 1984). The enzyme activity was lost after the enzyme-containing gel was soaked in H_2O_2, which indicated that this was an Fe-SOD enzyme (Jurtshuk et al. 1984).

The control strain 9043, grown in media containing 0 to 1 µM Fe^{3+}, failed to demonstrate catalase or SOD activity (Table 2.3). Unlike strain 184, strain 9043 grew well in these media and whole cells gave a rapid positive reaction in the H_2O_2 spot test for catalase. These results suggested that the cell extracts contained a material that interfered with the catalase and SOD assays. Dialysis of strain 9043 cell extract prepared from cells grown with 10 µM Fe^{3+}, increased the catalase and SOD specific activities twofold, but dialysis did not restore the activities in the other extracts. No similar interference was detected when other strains were used.

A. vinelandii was used as an alternative control. This strain had active catalase, ODPX, and SOD enzymes under iron starvation conditions (Table 2.3). SOD activity

Table 2.3 Effect of iron content of the growth medium on the activities of catalase, peroxidase, and SOD

Organism	Iron (μM)[a]	Enzyme specific activity[b]			
		Catalase	ODPX	PDPX	SOD
A. chroococcum					
9043	0	INT[c]	0.02	ND[d]	INT
	1	INT	0.04	ND	INT
	10	950	1.04	ND	157
184	0	ND	0.01	ND	87
	1	0.6	0.20	0.01	61
	10	2.7	0.37	1.90	79
A. vinelandii	0	99	0.71	ND	8
	1	95	0.24	ND	39
	10	49	0.43	ND	67

[a] Iron content of pregrowth medium.

[b] Expressed as units (milligram of protein)$^{-1}$ (see Materials and Methods).

[c] INT, interference in the assay.

[d] ND, not detected.

was notably increased by iron availability. Catalase and ODPX specific activities appeared to decrease at higher iron contents, possibly because the enzyme protein constituted a smaller percentage of the total cell protein.

2.3.6 Attempts to enhance catalase and peroxidase activities

The catalase activity in strain 9043 was maximum after 48 hours of incubation. The catalase activity was very low at 24 hours (0.8 U mg of protein^{-1}) and increased to as much as 2,400 U (mg of protein)$^{-1}$ at 48 hours. Strain 184, on the other hand, demonstrated similar low specific activities at 24 and 48 hours and maximum peroxidase activity at 24 hours.

The activities of strain 184 catalase and peroxidase were not affected significantly by the addition of 1 μM Ni^{2+}, Mn^{2+}, Zn^{2+}, Co^{2+}, Cr^{2+}, VO_3^{2-}, or Al^{3+} which were added individually to iron-sufficient medium. Thus deficiency of a trace metal ion essential for enzyme activity was not apparent.

When strain 184 was subjected to oxygen stress in iron-sufficient medium containing ammonium acetate, cell growth remained the same as that obtained with lower aeration, peroxidase and SOD activities decreased 50 to 60%, and catalase activity was undetectable. Poorly aerated, nitrogen-fixing cells had a relatively high catalase activity (10.3 U mg of protein^{-1}), SOD (115 U mg of protein^{-1}), and PDPX (1.31 U mg of protein^{-1}) activities. Oxygen stress of nitrogen-fixing cells increased growth 1.7-fold and increased PDPX activity twofold. Catalase and SOD activities, however, decreased to 17 and 22% respectively, of the low aeration values.

2.3.7 Microaerobic growth.

Under nitrogen-fixing conditions, strain 184 was able to grow in static cultures and produced turbidity throughout the medium after 3 or 4 days of incubation. There was no growth of *A. vinelandii* or *Azomonas macrocytogenes* and strain 9043 grew very poorly in static cultures. When ammonium acetate was present, the growth of strain 184 also was strictly aerobic and there was minimal growth in static cultures.

Similarly, strain 184 produced a zone of subsurface growth in tubes of Burk's nitrogen-free medium containing 0.05% agar. Strain 184 also produced initial subsurface growth in tubes of nitrogen-free NFb medium. When a low inoculation density (3.8×10^4 cells/tube) was used, the zone of initial growth was 5 mm below the surface after 44 hours of incubation and moved to the surface by 62 hours. With a higher inoculation density (3.8×10^7cells/tube), the initial growth was 2 mm below the surface at 15 hours, moved up 1 mm by 20 hours, and was at the surface by 44 hours.

Strain 184 from nitrogen-free static cultures was very difficult to recover by plate count assay. The recovery of viable cells on filter-sterilized or charcoal-containing

medium was only 12 to 19% of the total count. This recovery was only marginally greater than the 10% recovery obtained on normal autoclaved medium, which suggested that these cells were injured prior to plating despite attempts to avoid excess aeration during the mixing and dilution steps in the assay.

Nitrogen-fixing strain 184 from static cultures was more sensitive to H_2O_2 than cells similarly grown with ammonium acetate (Fig. 2.4). Increased aeration resulted in increased H_2O_2 toxicity. In each case, nitrogen-fixing cells were the most sensitive, but at 225 rpm, nitrogen-fixing cells were as sensitive as NH_4^+-grown cells. As noted earlier, this aeroadaptation of nitrogen-fixing cells did not correlate with higher levels of enzymes to provide protection from toxic oxygen products. Some of the increased toxicity with the increased aeration could have been due to additional toxic oxygen products generated in the medium during the incubation period. Short-term H_2O_2 treatments followed by viability measurements were not practical, however, because of the difficulty in recovering viable cells by plate count assays.

2.3.8 Effect of dihydroxybenzoic acids, charcoal, benzoic acid and copper on growth and nitrogen-fixation

Dihydroxybenzoic acids are known to enhance the aerotolerance of microaerophilic organisms such as *S. volutans* and *C. jejuni* (Krieg and Hoffman, 1986). In the presence of 2,3-Dihydroxybenzoic acid (10 μM) and 3,4-Dihydroxybenzoic acid (10 μM) neither the growth nor nitrogen fixing ability of strain 184 was stimulated (data not shown). Dithiothreitol completely inhibited the growth of the organism which therefore eliminated the requirement for absolute anaerobic growth conditions for strain 184. In the presence of copper (2.5 μM) or benzoic acid (1 mM), neither growth nor nitrogenase specific activity were affected (data not shown).

2.3.9 Effect of iron on growth under varying aeration conditions

Since strain 184 demonstrated microaerophilic characteristics, the iron requirement of the organism was studied under different aeration conditions since iron is an essential requirement for components of the electron transport system, nitrogenase activity and for protection of the oxygen-labile nitrogenase (Yates and Jones, 1974). The growth of strain 184 was significantly increased in the presence of 10 μM ferric citrate both under static growth conditions and under conditions of rotation at 250 rpm. However, the extent of increase was significantly higher under rotatory conditions (Fig. 2.5). 10 μM ferric citrate caused a 16-fold increase in growth of cells growing under rotatory growth conditions and only a 4-fold increase under static conditions. Above 10 μM the growth of the organism was not significantly increased under both growth conditions.

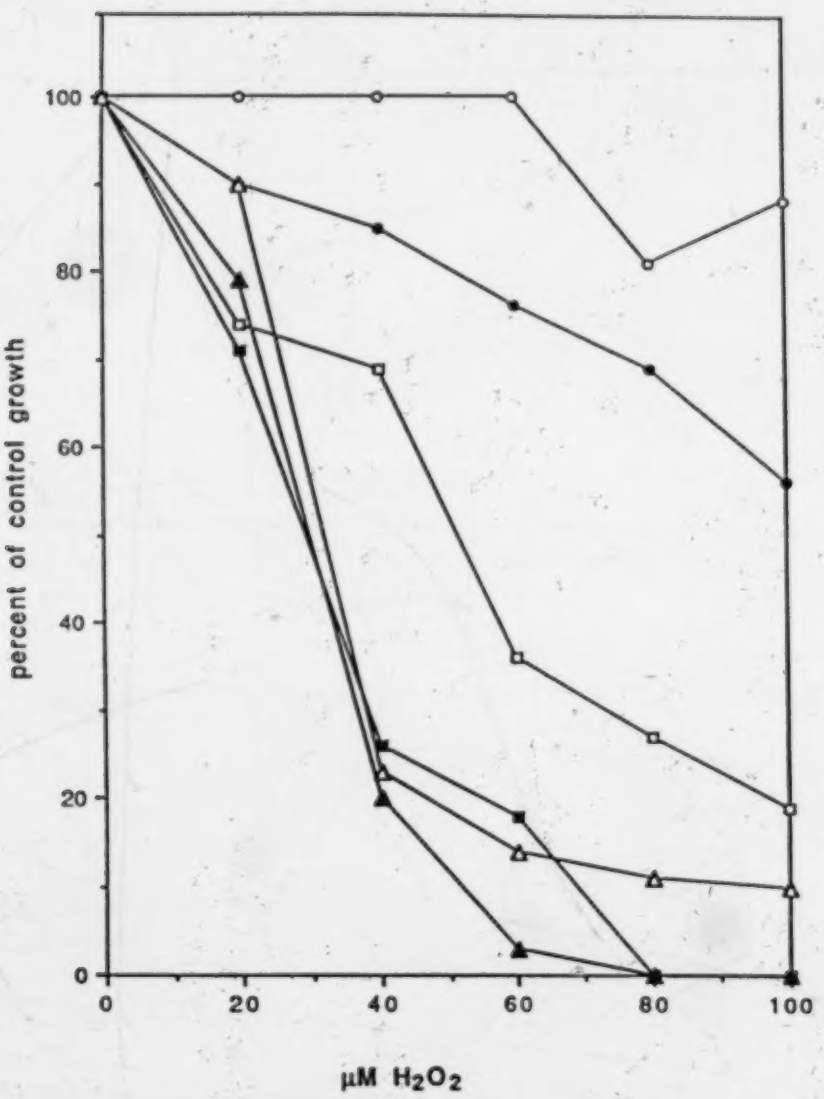

Fig. 2.4. Effects of aeration and nitrogen source on the sensitivity of strain 184 to H_2O_2 toxicity. Growth was calculated as in Fig. 2.1. The medium contained ammonium acetate (open symbols) or was nitrogen-free (closed symbols). Shaking speeds were adjusted to 225 rpm (△,▲) or 100 rpm (□,■) or were static (○,●).

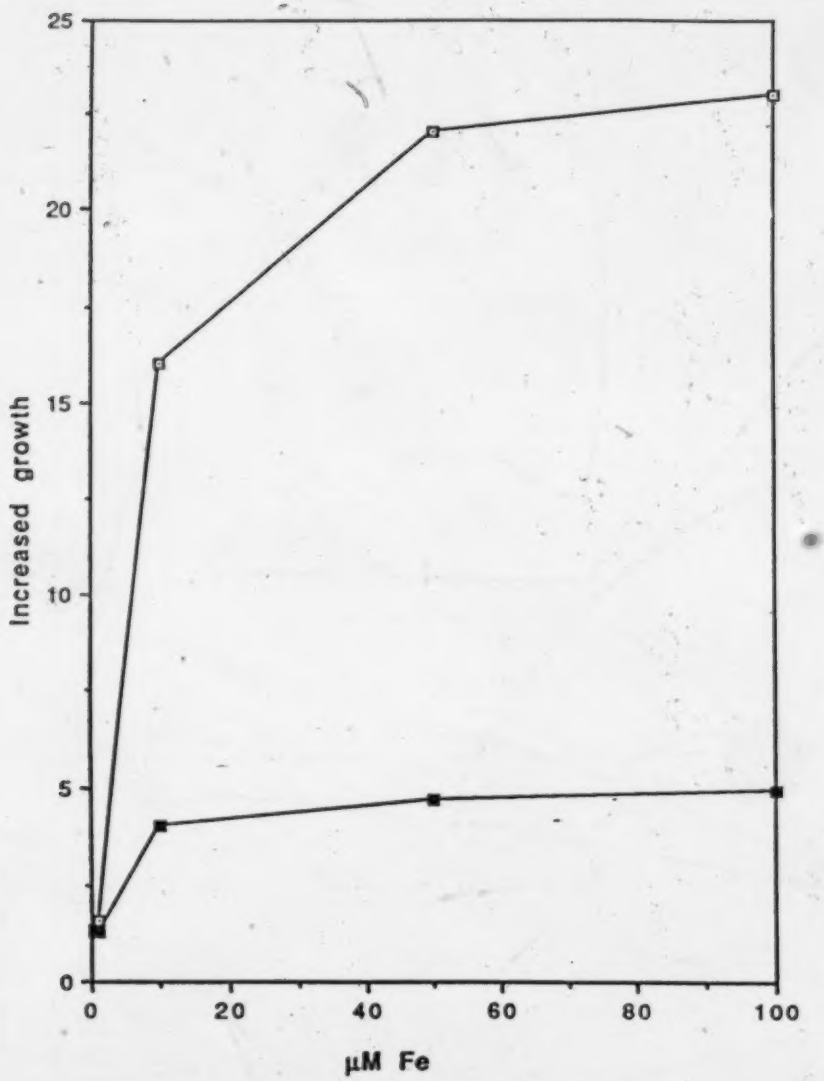

Fig. 2.5. Effect of iron on the growth of strain 184 under rotatory (⧈) and static conditions (■). Growth (micrograms of protein per milliliter measured after 52 hours of incubation in medium containing 10 µM ferric citrate and 2% sucrose) is shown as the fold-increase in growth over that at 0 Fe. Protein was measured by the Bradford method (1976).

2.3.10 Effect of initial head space O_2 concentration and carbon source on nitrogenase activity

Nitrogenase activity was optimal for strain 184 at 10% O_2 (initial head space concentration) rather than in 20% or 5% O_2 in the presence of 0.5% pyruvate as the carbon source (Fig. 2.6). Nitrogenase activity peaked around 16 hours. 0.5% pyruvate supported maximum nitrogenase activity at 10% O_2 as noted earlier, and above that, the activity decreased. However, in the presence of sucrose, the maximum nitrogenase activity was observed between 16 to 18% O_2. Interestingly, the maximum level of nitrogenase activity was approximately the same at the respective optima for O_2 (Fig. 2.6). With pyruvate as the carbon source, there was a significant drop in nitrogenase activity at 20% O_2 (about 4% of that at 10% O_2), whereas with sucrose, the same drop was not seen (was 50% of that at 18% O_2). This drop with pyruvate as carbon source was higher than seen in other studies (Chapter 3), although the levels of protein at both the oxygen levels were equivalent in both studies.

2.3.11 Effect of aeration on nitrogenase activity

A. chroococcum strain 184 demonstrated a definite preference for low aeration for maximum nitrogenase specific activity. As seen in Table 2.4, maximum nitrogenase activity was seen when the organism was grown at 150 rpm and not at 250 rpm. The increase in specific activity at low aeration was also more significant than for strain 9043. In strain 9043 in the presence of sucrose as the carbon source, there was only a 15% increase in nitrogenase activity with decreasing aeration, except under static conditions. However, with strain 184, there was a 72% increase with decreasing aeration except under static conditions. In the presence of pyruvate, the increase in nitrogenase activity at lower aeration was phenomenal in strain 184, whereas, in strain 9043, the increase was only 21% over that when the organism was grown at 200 rpm, there being no detectable activity at 250 rpm. As seen earlier with the effect of O_2 on nitrogenase specific activity, pyruvate supported optimal nitrogen fixation at low aeration in strain 184, there being negligible activity at the higher aeration. Growth was higher in the presence of sucrose as carbon source in both strains than pyruvate (Table 2.4) except with strain 184 under static growth conditions. As seen in Table 2.4, *A. chroococcum* strain 184 showed higher nitrogenase activity than strain 9043 when grown with pyruvate and sucrose as carbon source under static growth conditions. Strain 9043 demonstrated only 38 to 40% of the activity of that of strain 184 under these conditions.

The effect of aeration on nitrogenase activity was studied further by varying conditions during growth and during the assay. As seen in Table 2.5, nitrogenase activity was the highest when the organism was grown at 100 rpm rather than at 250

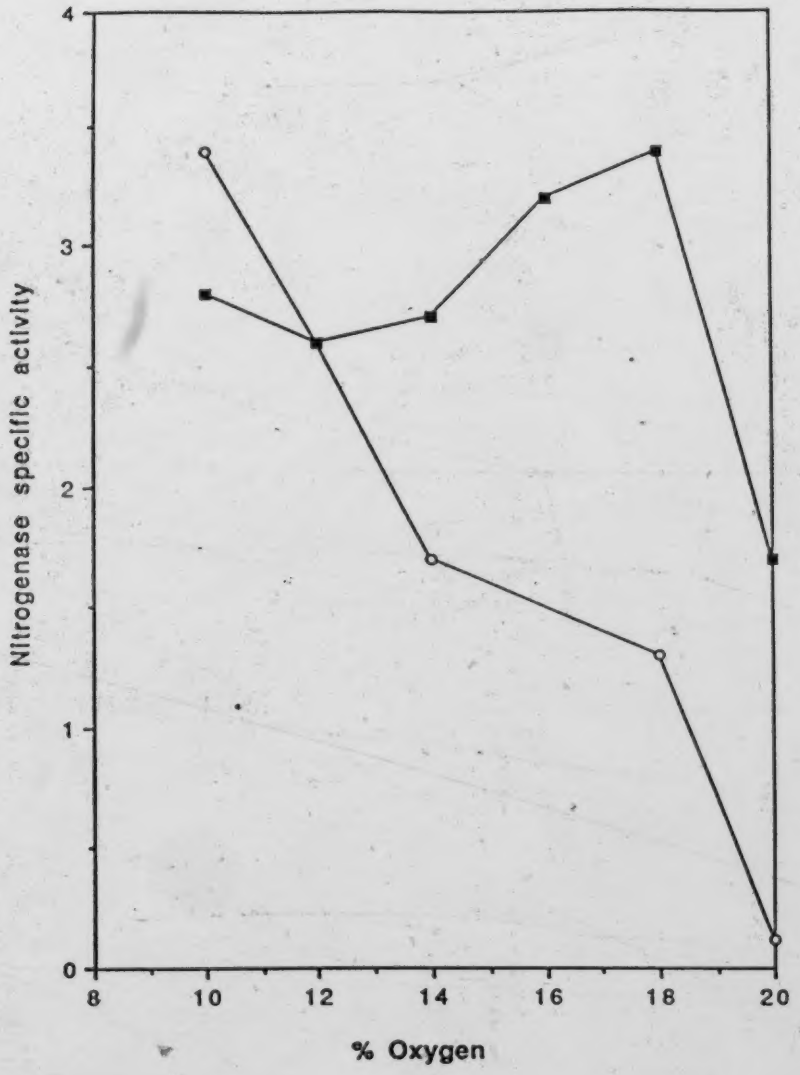

Fig. 2.6. Effect of initial head space oxygen on nitrogenase specific activity in strain 184 in the presence of 0.5% pyruvate (o) and sucrose (■). Nitrogenase activity (micromoles ethylene produced per milligram protein per hour, was measured after 24 hours incubation in 160-mL serum bottles in Burk's medium containing 10 μM ferric citrate). Protein was estimated by the Bradford's method and nitrogenase activity was estimated as described in Materials and Methods.

Table 2.4 Effect of aeration on growth and nitrogenase activity of *A. chroococcum* strains 184 and 9043[a]

Carbon source/aeration	Protein[b]		Nitrogen fixation[c]	
	184	9043	184	9043
Sucrose (0.5%)				
Static[d]	17	43	1.40	0.67
150 rpm	200	153	2.96	3.00
200 rpm	208	160	2.96	2.70
250 rpm	253	179	1.72	2.60
Pyruvate (0.5%)				
Static[d]	43	18	2.1	0.79
150 rpm	124	112	2.5	5.70
200 rpm	168	143	0.002	4.70
250 rpm	25	126	0.01	0.00

[a] All values are means of at least two duplicate determinations. Samples were withdrawn from the cultures (15 mL per 50 mL Erlenmeyer flask) after 24 hours incubation except for static cultures. Nitrogenase activity was measured as described in Materials and Methods.

[b] Protein was estimated by the method of Bradford (1976) and was expressed as microgram protein milliliter^{-1}.

[c] Expressed as micromoles ethylene produced (milligram of protein)$^{-1}$ hour^{-1}

[d] Samples were withdrawn after 48 hours incubation.

Table 2.5 Effect of aeration on growth and nitrogen fixation under different assay conditions[a]

Aeration during growth	Incubation period (h)	Nitrogenase activity[b]		
		250 rpm	100 rpm	static
—				
250 rpm	26	4.50	ND	0.13
	52	2.00	ND	0.05
100 rpm	26	5.7	1.15	0.95
	52	6.20	0.67	1.00
Static	52	3.00	2.71	0.83

a All values are means of at least two duplicate determinations. Samples were withdrawn from the cultures (15 mL per 50 mL Erlenmeyer flask) at the time intervals mentioned. Sucrose (2%) was used as the carbon source. Protein was estimated by the method of Bradford (1976). Nitrogenase activity was measured as described in Materials and Methods.

b Expressed as micromole ethylene produced (milligram of protein)$^{-1}$ hour^{-1}

ND Not determined

rpm. Interestingly, the specific activity was higher when the assay was carried out in the sealed vials at higher agitation than at the lower agitation. It is possible that if the assay was carried out at later time intervals with statically-grown cells, the activity may have been higher, since the organisms have a longer lag phase and lower growth rate under static growth conditions (data not shown). When cells were grown in medium containing 0.05% agar in 13 mm test tubes under static and rotatory conditions, it was noticed that there was about a 4-fold increase in nitrogenase specific activity under rotatory conditions than under static conditions when sucrose was the carbon source (data not shown). The agar would help in reducing the diffusion of oxygen into the medium and thereby keep the level of dissolved oxygen in the medium at a low level. With pyruvate, the maximum nitrogenase activity was seen under static conditions in liquid culture rather than shaking conditions, there being not much of a difference when grown microaerobically with and without shaking (data not shown). These results further confirm the requirement for oxygen for growth and nitrogen fixation for strain 184 but at a level lower than that present at normal atmospheric levels.

2.3.12 Carbon source requirement under varying aeration conditions

Strain 184 was grown with carbohydrates and organic acids under microaerobic conditions and aerobic conditions to study nitrogenase specific activity and growth. As seen in Table 2.6, growth was stimulated in the presence of gluconate as the sole carbon source under aerobic conditions. Under microaerobic conditions, on the whole, organic acids supported the highest nitrogenase specific activity, the highest being seen with succinate and pyruvate, although higher levels of growth were seen with carbohydrates, in particular melibiose and maltose. Mannitol also supported high nitrogen fixing ability under microaerobic conditions. Under aerobic conditions, the carbohydrates supported the best growth and nitrogen-fixing ability. Among these sucrose, galactose, mannitol and maltose supported the highest nitrogen fixing activities. Among the organic acids, lactate supported high nitrogenase activity, equivalent to that shown by the carbohydrates. In the presence of citrate and α-ketoglutarate growth was negligible. The low specific activity of nitrogenase in gluconate-grown cells could be due to the time at which samples were analyzed, since growth was already so high at 22 hours and the organism could have become oxygen limited.

When grown in the presence of ammonium, gluconate and maltose supported the maximum growth under aerobic conditions (data not shown). Using semisolid media, the cells grew as a pellicle on the surface of the medium, unlike under nitrogen-fixing conditions, and maltose and melibiose supported the maximum growth (data not shown).

Table 2.6 Growth and nitrogen-fixation under microaerobic and aerobic conditions using various carbon sources[a]

Carbon source[b]	Protein		Nitrogen fixation[c]	
	Aerobic	Microaerobic	Aerobic	Microaerobic
Citrate	2	9	9	40
Gluconate	224	19	3	80
α-KG	3	13	18	2
Lactate	67	14	1330	57
Malate	64	14	<1	52
Pyruvate	121	14	<1	95
Succinate	61	11	11	114
L-Arabinose	24	25	2	38
D-Galactose	51	23	990	44
D-Glucose	79	15	650	77
Maltose	111	31	940	50
Mannitol	95	14	940	93
D-Mannose	30	25	2	30
Melibiose	54	37	610	17
Sucrose	122	25	1000	30

[a] All values are means of at least two duplicate determinations. Samples were withdrawn after 22 hours incubation from the cultures (15 mL per 50 mL Erlenmeyer flask for aerobic conditions and 5 mL containing 0.5% agar per 13 mm test tube for microaerobic conditions). Samples were analyzed as described in Materials and Methods. Protein was estimated by the method of Lowry et al. (1951) and was expressed as microgram protein milliliter^{-1}.

[b] The carbon source was provided at 30 mM concentration during aerobic conditions and 10 mM for microaerobic growth.

[c] Expressed as nanomoles ethylene produced (milligram of protein)$^{-1}$ hour^{-1}

2.3.13 Plasmid content of strain 184

Plasmid preparations of strain 184 were carried out by several methods and the organism was found to harbour a plasmid (Fig. 2.7). Among the methods used that of Robson et al. (1984) proved to be the most efficient. Restriction analysis of the plasmid was carried out, and it was noted that *Cla*I, *Eco*RI, *Nco*I, *Pst*I and *Sst*I sites were present on the plasmid (data not shown). There were no sites for *Bam*HI, *Bcl*I, *Bst*EII, *Hind*III and *Pvu*II. The size of the plasmid was estimated to be between 30 and 35 kb by restriction analysis. Using the multiple plasmid-containing *E. coli* strain V517, the size of the plasmid was estimated to be about 28 kb (Fig. 2.8).

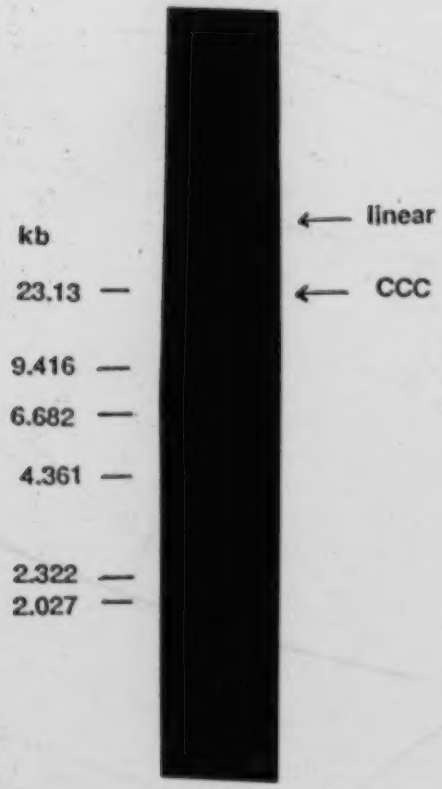

Fig. 2.7. Plasmid in strain 184 (indicated by arrows). The plasmid was isolated by the procedure of Robson et al. (1984) as described in Materials and Methods. The positions of the size standards (lambda DNA digested with *Hind*III) are shown.

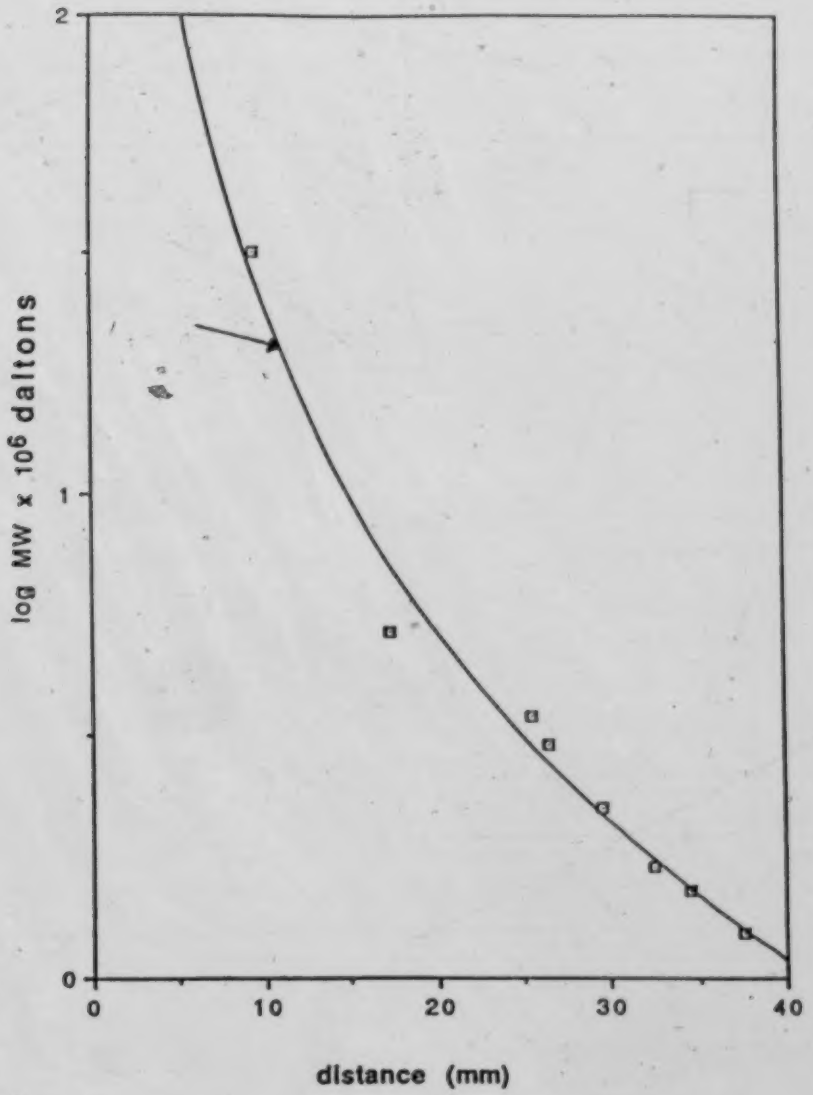

Fig. 2.8. Migration of CCC molecules of plasmids found in *E. coli* V517 versus the logarithm of the molecular weight, on analysis by vertical agarose gel electrophoresis. The logarithmic curve fit is shown. The plasmids have sizes of (x 10^6 daltons): 32.04 ± 3.06, 5.19 ± 0.27, 3.48 ± 0.20, 3.03± 0.18, 2.24 ± 0.09, 1.69 ± 0.10, 1.51 ± 0.09, 1.24 ± 0.09. The arrow indicates the position of the CCC form of strain 184 plasmid.

2.4 DISCUSSION

Na^+-dependent *A. chroococcum*, represented by strain 184, appear to be aeroadaptive microaerophilic bacteria. This growth relationship with oxygen is very unusual and seems to be unique within the *Azotobacteraceae* family. The results show that initial nitrogen-fixing growth can start at reduced oxygen concentration and that as the cell density increases, the growth moves to a more aerobic environment. Cells that are not nitrogen-fixing grow as aerobes only. This pattern of growth has also been observed with other nitrogen-fixing microaerophilic bacteria, for example, *Rhizobium* and *Azospirillum* spp. (Dobereiner and Pedrosa, 1987; Keister, 1975; Nelson and Knowles, 1978). With *Azospirillum* spp., there is a precise requirement for a low dissolved-oxygen concentration during initial growth. As the cell density increases, the oxygen demand increases and the cells move toward the surface of the medium (Dobereiner and Pedrosa, 1987). However, both *Rhizobium* and *Azospirillum* spp. fix nitrogen only under microaerobic conditions and do not demonstrate respiratory protection of the nitrogenase (Keister, 1975; Nelson and Knowles, 1978; Okon et al. 1976). Na^+-dependent *A chroococcum*, on the other hand, fix nitrogen under aerobic conditions in batch cultures well enough to appear to be aerobes (Page, 1986).

An interesting group of *A. chroococcum* mutants with an oxygen-sensitive, nitrogen-fixing phenotype has been described by Ramos and Robson (1985). These bacteria are unable to fix nitrogen aerobically with sugars (Fos^-) and grow as a zone 2 to 3 mm below the surface of medium gelled with 0.5% agar. Aerotolerant nitrogen-fixation was restored with the use of carboxylic acids rather than sugars, which suggested that the defects in sugar catabolism were preventing the operation of respiratory protection (Robson and Postgate, 1980; Ramos and Robson, 1985). Whereas these mutants are interesting, they bear little resemblance to the naturally occurring Na^+-dependent *A. chroococcum*. The latter has no difficulty growing aerobically with N_2 and sugars (Page, 1986; this study) and express the microaerophilic growth characteristic without the Fos^- phenotype. Also, microaerobic growth of strain 184 could not be demonstrated with 0.5% agar in the medium (data not shown).

Robson et al. (1985) report the requirement for low oxygen tensions (0.2% to 1% O_2) for the Fos^- mutants to demonstrate nitrogen-fixing growth. Strain 184 demonstrated nitrogen-fixing ability even at 5% O_2 in the head space although the optimal level of O_2 was 10% (in the head space)with pyruvate as carbon source and 16-18% (in the head space) with carbohydrates. Drozd and Postgate (1970) studied the effect of oxygen on acetylene reduction, cytochrome content and respiratory activity in *Azotobacter*

chroococcum. They observed that the maximum acetylene- reducing activity of cultures of low respiratory activity and high respiratory activity were similar at their optimal p_{O2} values. In their study using continuous cultures, with mannitol as the carbon source, when the organism was grown at p_{O2} of 0.09 atm the optimum for dissolved oxygen for acetylene reduction was 12 μM, whereas for a culture grown at p_{O2} 0.55 atm, the optimum was at 25 μM (Drozd and Postgate, 1970). Thus the organism appeared to have adapted to various oxygen tensions. Strain 184 appears to fix nitrogen under low and high oxygen tensions depending on the carbon source, although its preference is for the lower levels of oxygen. When strain 184 was grown under static conditions or at 100 rpm, however, it did show higher nitrogenase specific activity at the higher aeration. The specific activity for acetylene reduction was higher in shaken cultures as compared to stagnant cultures, similar to that seen in *A. lipoferum* (Okon et al. 1976). This could be due to an increased contact between the cells and the reactants in the assay under shaking conditions.

In *Azotobacter* species, in addition to the nitrogenase itself being an oxygen-sensitive component, electron carriers to nitrogenase, viz. ferredoxins and flavodoxins are also oxygen sensitive (Robson and Postgate, 1980). Autooxidation of flavodoxin hydroquinone, which is the primary electron donor to nitrogenase in *Azotobacter*, could result in the production of superoxides in the cell (Robson and Postgate, 1980). Thus, although azotobacters are obligate aerobes, the efficiency of nitrogen fixation decreases with increasing aeration (Dalton and Postgate, 1969). This is because azotobacters respire at a rate which results in a very rapid consumption of oxygen to maintain its nitrogenase in an essentially anoxic environment inside the cells. This is the respiratory protection which has been well documented in azotobacters (Dalton and Postgate, 1969). A second means of protection becomes important when oxygen enters the culture more rapidly than it is removed, giving rise to an O_2-stable nitrogenase complex caused by the binding of a 2Fe-2S protective protein (Robson, 1979). At very high levels of oxygen there is a repression of nitrogenase synthesis (Robson, 1979). An additional response to increased oxygen levels is the formation of intracytoplasmic membranes, which are thought to be involved in increasing the respiratory capacity of the organism (Post et al. 1983). Thus organisms belonging to the genus *Azotobacter* have evolved various mechanisms to protect themselves from the toxic effects of oxygen. However, strain 184 appears to be unusual in this respect, because it demonstrates a preference for lower levels of aeration and oxygenation.

A study of the carbon source requirement under different aeration condition demonstrated a preference for organic acids under microaerobic conditions. This is

similar to the characteristics of the microaerophilic nitrogen-fixer *Azospirillum* (Dobereiner and Pedrosa, 1987). Among the organic acids citrate and α-ketoglutarate did not support growth and nitrogen-fixation under aerobic and microaerobic conditions. Although citrate utilization has not been reported in *Azotobacter* (Thompson and Skerman, 1979), except in the case of the Fos^- mutants of Robson et al. (1985), α-ketoglutarate utilization is reported by all *Azotobacter* strains (Thompson and Skerman, 1979). Thus the inability of strain 184 to utilize α-ketoglutarate is unusual. Among azospirilla, *A. brasilense* and *A. amazonense* do not utilize α-ketoglutarate, but *A. lipoferum* does (Dobereiner and Pedrosa, 1987). In the presence of gluconate growth was very high in strain 184. *Azotobacter* metabolizes glucose principally via the Entner Doudoroff (ED) pathway (Mortenson and Wilson, 1954; Mortenson et al. 1955; Stephenson et al. 1978). Harris (1946) observed that glucose-grown cells of *A. chroococcum* oxidized gluconate faster than glucose itself and thus the increased growth of strain 184 is probably due to the fact that gluconate is an intermediate of the ED pathway. The lower levels of nitrogenase specific activity with gluconate under aerobic conditions can be explained by two reasons, viz. firstly because of oxygen limitation during the assay since growth was high and, secondly, because the carbon source is gluconate, the energy efficiency and level of reducing equivalents is lower than for carbohydrates. L-arabinose and D-mannose do not support growth of any *Azotobacter* species (Thompson and Skerman, 1979) and strain 184 also showed a similar inability to metabolize these carbon sources. Under microaerobic conditions melibiose did not support high levels of nitrogen fixation in strain 184, unlike that seen under aerobic conditions. Mannitol was a good carbon source under both aerobic and microaerobic conditions. The metabolism of mannitol is thought to proceed via fructose since a mannitol dehydrogenase has been reported in *A. chroococcum* (Jensen, 1954). Thus strain 184 grows and fixes nitrogen microaerobically best with the organic acids succinate and pyruvate and in the presence of mannitol. Under aerobic conditions, lactate, maltose, mannitol and sucrose support the highest nitrogen fixation at atmospheric oxygen levels.

True to their microaerophilic nature, strain 184 and other Na^+-dependent strains have only low catalase and peroxidase activities. As noted by Krieg and Hoffman (1986), many microaerophilic bacteria lack or have low levels of catalase activity. SOD activity is present in most microaerophiles and would appear to be less involved in determining microaerophily than catalase activity (Hoffman et al. 1979; Krieg and Hoffman, 1986). In strain 184, the activities of these enzymes were enhanced by iron availability. The SOD of strain 184 also was demonstrated to be an Fe-SOD, as reported for other azotobacters (Jurtshuk et al. 1984). The very low activities of these enzymes in

iron-starved cells and the sensitivity of strain 184 to toxic oxygen products explains the inability of these strains to grow under iron-starvation conditions (Page, 1987). The small amount of iron required by these strains before growth under iron starvation conditions is probably required to activate the enzymes involved in protection against toxic oxygen products. Similarly, the carry-over of iron after shift-down of cells from iron-sufficient to iron starvation conditions would appear to provide enough active enzymatic protection to permit growth and hydroxamate siderophore production (Page, 1987).

Attempts were made to purify the hydroxamate siderophore from culture supernatants of strain 184 using charcoal as adsorbent and LH-20 columns. Optimal conditions for hydroxamate production were the use of Fe_3O_4 as iron source and mannitol or sucrose as the carbon source. Interestingly, mannitol has been found to be the preferred carbon source in the production of the fluorescent siderophore, azoverdin, in *Azomonas macrocytogenes* (Collinson and Page, 1989). The increased production of siderophores in the presence of mannitol in strain 184 may be due to its iron binding ability (Gutteridge, 1987) thereby mediating the entry of low levels of iron to initiate growth and siderophore production. Initial characterization identified the hydrophilic nature of the compound and the possible presence of an amino acid moiety. Iron uptake assays on culture supernatants demonstrated the ability of the siderophore to mediate iron assimilation. Interestingly, citrate which is known to be excreted by the organism (Page, 1987) also mediated uptake. Thus as seen in *E. coli* K-12 (Hussein et al. 1981), citrate must be involved in low affinity iron uptake in this Na^+-dependent strain as has been suggested earlier (Page, 1987). Among the other iron chelators NTA also mediated iron uptake in strain 184. *E. coli* mutants unable to produce siderophore are known to be able to obtain iron from media containing this compound (Frost and Rosenberg, 1973). During the course of the studies on hydroxamate production it was also noted that nitrogen-fixing cells turned brown to black after 4 days of growth, the same appearance being absent in ammonium-grown cells.

Even under iron-sufficient conditions strain 184 appears to suffer cell injury such that only $\leq 44\%$ of the population can be recovered. Recovery is not enhanced by protection from H_2O_2 or atmospheric O_2, indicating that this damage occurred before the cells were plated. This will contribute to the difficulty in isolating these bacteria from the soil. It is interesting that a large number of Na^+-dependent strains were isolated by F.D. Cook by using enrichment in tubes of nitrogen-free medium and incubation under static conditions (Page, 1986). The soil-sprinkling plate method of Winogradsky (Becking, 1981), on the other hand, has produced only a few new Na^+-dependent strains from the

soils of Alberta and Saskatchewan (W. Page, unpublished data). The results of this study now indicate how to proceed in the development of methods for the isolation of these bacteria. These methods would involve growth under microaerobic conditions or by using growth media treated to decrease their free radical content.

The size of the plasmid present in *A. chroococcum* strain 184 is about 19-22 MDal (30-35 kb). The role of the plasmid in strain 184 is not known. In a study of plasmids present in eight strains of *A. chroococcum*, all eight were found to harbour between 2 and 6 plasmids ranging from 7 to > 200 MDal (Robson et al. 1984). No definite function has been assigned to these plasmids. In another study by Saitia et al. (1989), plasmids of *A. chroococcum* were thought to be involved in conferring heavy metal resistance to the organism. *Azospirillum* species are also known to harbour indigenous plasmids although no specific phenotype has been conclusively found to be plasmid-borne (Dobereiner and Pedrosa, 1987). In various *Rhizobium* strains the ability to produce melanin is encoded on plasmids (Lamb et al. 1982; Cubo et al. 1988; Hynes et al. 1988). The investigation into the role of the plasmid in strain 184 should be interesting.

It is apparent from these studies that *A. chroococcum* 184 has very little capacity to adjust the activities of protective enzymes to aeration, nitrogen fixation, or toxic oxygen stress. The sensitivity of this strain to toxic oxygen generated in normal plating media also would not be expected if these bacteria could adjust enzyme levels to afford protection when challenged. This sensitivity to normal media is a classic problem encountered in the isolation, growth, and enumeration of microaerophilic bacteria (Krieg and Hoffman, 1986). Strain 184 was notably sensitive to H_2O_2, being inhibited 50% by 40 μM H_2O_2. Another catalase-positive microaerophile, *Campylobacter jejuni*, is similarly sensitive to 35 μM H_2O_2 (Hoffman et al. 1979).

Dihydroxyphenyl compounds stimulate the growth of microaerophilic organisms such as *Spirillum volutans* and *Campylobacter fetus* subspecies *jejuni* (Bowdre et al. 1976). The ability of these compounds to stimulate growth was at first attributed to their iron binding ability and therefore increasing iron availability to the organisms (Bowdre et al. 1976). However, it is now known that these compounds are able to increase the aerotolerance of microaerophiles because of their ability to destroy superoxide radicals (Hoffman et al. 1979).

Both strains 184 and 9043 were very sensitive to paraquat. Interestingly, iron-sufficient cells were more sensitive than iron-limited cells. As shown by Korbashi et al. (1987), iron also mediates the toxicity of paraquat in *E. coli*, whereby not only does paraquat generate $O_2^{\cdot}$ by reaction of O_2 with the paraquat radical but also Fe^{3+} bound to

biomolecules may participate in a form of redox cycling to generate ·OH by a site-specific Fenton reaction. Hasset et al. (1987) also have suggested that paraquat toxicity is due to extracellular production of $O_2^{\cdot-}$, in reaction with O_2, and intracellular generation of ·OH.

Some microaerophiles and all anaerobes are susceptible to metronidazole, which is toxic only when it is reduced (Krieg and Hoffman, 1986). This compound causes single- and double-stranded breaks in DNA and a loss of DNA helicity (Edwards, 1977; Knight et al. 1978). It is also thought to act as an electron sink (Lockerby et al. 1984). The selective toxicity of metronidazole to anaerobes is linked to the preference for ferredoxin and flavodoxin for metronidazole reduction (Müller, 1983; Lockerby et al. 1984; Tally et al. 1978; Edwards, 1977). Since azotobacters have flavodoxins which are involved in electron transport to nitrogenase (Hagër et al. 1988), both the *A. chroococcum* strains 184 and 9043 and the *A. vinelandii* UW strain demonstrated sensitivity to metronidazole. The *A. chroococcum* strains demonstrated a higher sensitivity than the *A. vinelandii* strain. A 96% and 83% reduction in growth was seen with 50 μg metronidazole mL^{-1} with *A. chroococcum* strain 9043 and 184, respectively. The microaerophile *S. volutans* is completely inhibited by 0.05 μg metronidazole mL^{-1}, whereas organisms of the genus *Campylobacter* show a range of sensitivity between 0.25 to 64 μg mL^{-1}. The sensitivity of azotobacters to metronidazole indicates that there is a low redox potential present in the cells in order to maintain the nitrogenase in an active form.

Under aerobic conditions, strain 9043 would presumably resist $O_2^{\cdot-}$-mediated killing by dismutation of the $O_2^{\cdot-}$ to H_2O_2 and removal of the latter with catalase. The removal of H_2O_2 would then prevent the internal iron-catalyzed Fenton reactions. In strain 184, $O_2^{\cdot-}$ would similarly be removed by SOD, but H_2O_2 may accumulate because of the low activity of catalase. This could be like the situation observed for SOD-rich *E. coli*, in which these cells are unusually sensitive to $O_2^{\cdot-}$ because of the excessive generation of H_2O_2 (Scott et al. 1987). Interestingly, *E. coli* with high SOD activity is also more readily killed by hyperoxia (Scott et al. 1987). In another situation, a mutant of *A. brasilense* which overproduces catalase did not show better resistance to inactivation and repression of nitrogenase by oxygen than the wild type (Dobereiner and Pedroasa, 1987). Therefore, aeroadaptation may be facilitated by the correct ratio of H_2O_2-destroying enzymes and SOD activity. With strain 184, aerobic stress of nitrogen-fixing cells resulted in a two-fold increase in peroxidase activity and an 83% decrease in SOD activity without a loss in cell yield. Peroxidases are thought to be important for microaerophiles because of the lack of or low scavenging ability of catalases for H_2O_2 (Krieg and Hoffman, 1986). If strain 184 were able to prevent the formation of toxic

oxygen products and H_2O_2, or be able to remove it from the oxygen-sensitive sites in the cell, it would explain its ability to grow and survive under aerobic conditions inspite of its microaerophilic characteristics.

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CHAPTER 3

Catechol formation and melanization by Na^+-dependent *Azotobacter chroococcum*: a protective mechanism for aeroadaptation?

3.1 INTRODUCTION

Organisms of the genus *Azotobacter* are free living nitrogen-fixing obligate aerobes (Becking, 1981). Among species of this genus, *Azotobacter chroococcum* has been most commonly isolated from the soils worldwide. Recently, strains of *Azotobacter chroococcum* absolutely dependent on Na^+ for growth have been isolated from the soils of Alberta, Canada (Page, 1986). These organisms have an iron-dependent growth phenotype (Page, 1987) and the iron dependence has been linked to the activity of catalase, peroxidase and superoxide dismutase (Chapter 2; Page et al. 1988). The activities of the former two enzymes are very low in the Na^+-dependent strains and these bacteria share many traits with microaerophilic bacteria. However, despite the low activity of these enzymes, these bacteria can grow and fix nitrogen aerobically, unlike other microaerophilic bacteria (Page, 1986; Page et al. 1988; Chapter 2).

We have become interested in the intense brown-black pigment formed by the Na^+-dependent strains. This pigment has been described, but not proven to be a melanin in *Azotobacter chroococcum* (Jensen, 1954; Thompson and Skerman, 1979). Melanins are formed by the oxidative polymerization of phenolic compounds such as catechol and 1,8-dihydroxynaphthalene (allomelanins) (Allport and Bu'Lock, 1958), DOPA (eumelanins) and cysteinyl-dopa (phaeomelanins) (Sarna et al. 1984). The eumelanins and phaeomelanins are found mainly in animal species, whereas, the allomelanins are found in plants and microorganisms (Nicolaus et al. 1964). Among microorganisms,

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melanin formation has been observed in *Micrococcus* (Mung and Kondrat'eva, 1981), *Bacillus* (Aronson and Wermus, 1965), *Streptomyces* (Arai and Mikami, 1972), *Vibrio* (Ivins and Holmes, 1980), *Mycobacterium* (Prabhakaran et al. 1968), *Rhizobium* (Beynon et al. 1980) and recently in an *Azospirillum* species (Sadasivan and Neyra, 1987), to name a few. Melanins are not considered essential for growth and development of cells but are required in order to enhance the ability of the producing species to compete and survive under certain environmental conditions such as in the presence of ultraviolet radiation (Bell and Wheeler, 1986). Sakina et al. (1980) describe antioxidant properties of melanin where lipid peroxidation is inhibited due to the ferrous ion-binding and antiradical activity of the pigment.

All aerobic organisms form the superoxide radical and hydrogen peroxide (Fridovich, 1978). Both $O_2^{\cdot}$ and H_2O_2 can do direct damage to cellular targets (Fridovich, 1978), but on the whole their reactivity is limited. However, in the presence of iron catalysts, $O_2^{\cdot}$ and H_2O_2 interact to form the highly reactive hydroxyl radical ($\cdot OH$) via the Fenton reaction (Gutteridge, 1987).

$$O_2^{\cdot} + H_2O_2 \xrightarrow{\text{Fe catalyst}} \cdot OH + OH^- + O_2$$

The enzymes catalase, peroxidase and superoxide dismutase are involved in protecting aerobic organisms by preventing the formation of these radicals (Jurtshuk et al. 1984). In animals, non-enzymatic antioxidants are also thought to play an important role in protection from oxidative stress. Among these compounds are uric acids and urates (Ames et al. 1981), vitamin C, plasma proteins (Wayner et al. 1985), vitamin E (Burton and Ingold, 1986) and β-carotene (Burton, 1984). Since the major damage to biological molecules is mediated by hydroxyl radicals, compounds which scavenge free iron and therefore make it unavailable to react in the Fenton reaction are potential candidates for free radical scavenger activity. This can be regarded as a significant part of aerobic antioxidant defense (Halliwell and Gutteridge, 1986). One of such compounds is melanin, a cation exchange polymer, which can protect tissues against oxidizing and reducing conditions and trap free radicals (Mason et al. 1960). Goodchild et al. (1981) have demonstrated the superoxide-scavenging ability of melanin, and Bell and Wheeler (1986) describe melanin as a "sponge" for free radicals which result from environmental stress. Korytowski et al. (1986) describe a pseudo-superoxide dismutase activity of a DOPA-melanin, indicating a further role in O_2 protection. The present study concerns the characterization of the melanin produced by *A. chroococcum* strain 184, the influence of

environmental conditions on catechol and melanin formation, and their possible role in allowing this microaerophilic organism to adapt to aerobic growth conditions.

3.2 MATERIALS AND METHODS

3.2.1 Organism, media and growth conditions

The Na^+-dependent strain of *Azotobacter chroococcum* strain 184 (Page, 1986) isolated from Alberta soils was used in this investigation. It was maintained on slants of Burk's nitrogen-free medium containing 0.85 mM NaCl. Liquid media, pH 7.2, contained 4.6 mM K_2HPO_4, 1.46 mM KH_2PO_4, 0.8 mM $MgSO_4 \cdot 7H_2O$, 1 μM $Na_2MoO_4 \cdot 2H_2O$, 13.2 mM $CH_3COONa \cdot 3H_2O$, and 0.453 mM $CaCO_3$. The nitrogen source was either 14 mM ammonium acetate or N_2 and 2% (w/v) sucrose was used as the carbon and energy source. The iron content of the medium was adjusted using ferric citrate. To study the effect of light on melanin formation, slants were illuminated by placing them two feet below a fluorescent lamp (15W cool white) while standing in a water bath at 30°C for 7 days.

Cultures (15 mL media per 50 mL Erlenmeyer flasks) were incubated under aerobic conditions at 30°C in a gyratory water bath shaker (New Brunswick Scientific Model G-76) at a shaking speed of 250 rpm in all the studies. Rates of aeration of nitrogen-fixing strain 184 were varied by using 5 to 30 mL volumes in 50 mL Erlenmeyer flasks. Cultures were inoculated using a 1% (v/v) inoculum as described previously (Page, 1987). Growth was monitored by estimating culture protein content as described by Bradford (1976) using bovine serum albumin (Sigma) as the standard. The cell pellet was resuspended in 1N NaOH and incubated in a 30°C water bath up to a maximum of 2 hours. Variability of up to 10% was observed in results obtained between replicate experiments.

3.2.2 Identification of catechol

The catechol produced by strain 184 was extracted from acidified (HCl, pH 2) growth supernatant fluid with ethyl acetate. The ethyl acetate extract was removed by rotary evaporation at 30-34°C. The residue was then dissolved in methanol:chloroform (5:95; v/v) and further purification was carried out by preparative thin layer chromatography (TLC) on silica gel-G plates using the same solvent mixture. The spray reagents used were ferric chloride: 2,2'-dipyridyl (Krebs et al. 1969) and 0.1 M $FeCl_3$ in

0.1 M HCl. Pure catechol, 2,3-Dihydroxybenzoic acid and 3,4-Dihydroxybenzoic acid were used as standards. The silica gel containing catechol was scraped from the plates and placed in a pasteur pipette, and the catechol was eluted with chloroform. The catechol purified from the culture supernatant fluids had the same R_f value as authentic catechol in all the solvent systems used: methanol:chloroform (5:95; 3:0.3; 3:1; 3:2; 1:1; 0.1:1; 0.2:1; v/v), benzene:acetic acid water (2:2:1; 2:3:1; 2:4:1; v/v), butanol:acetic acid:water (4:1:5; v/v), chloroform:acetic acid:water (140:8:52; v/v). Pure chloroform, benzene, methanol and ether also were used as solvents.

UV-visible wavelength scan of the purified catechol was carried out using a Perkin Elmer Lambda 3 spectrophotometer. The compound was conclusively identified as catechol using gas chromatography on a 30 m DB-1 capillary column with helium as the carrier gas at a flow rate of 45 cm/s and a holding temperature of 90°C coupled to mass spectrometric analysis. Pure catechol was used as a control.

Quantitative estimations of catechol in culture supernatant fluids was carried out using the Arnow procedure as modified by Barnum (1977). Authentic catechol was used as a standard. The A_{503} was read after 30 minutes of NaOH addition. Variability in results between replicate experiments was up to a maximum of 15%. For the identification of the Arnow-reactive compounds in NH_4^+-grown cells, DL-DOPA and L-tyrosine were subjected to the reaction and the reaction scanned to determine the wavelength of maximum absorption. DL-Dopa had an optimal reaction time of 2 minutes prior to the addition of NaOH, longer reaction times causing the colour produced to fade due to instability of the product.

3.2.3 Melanin estimation

The black pigment produced by the organism was identified as a melanin based on the properties described in White (1958). Melanin was purified from the cells by the method described by Whittaker (1963), where, the cell pellet was first extracted three times with 5% trichloroacetic acid, twice with ether: alcohol (1:1; v/v), and once with absolute ether, to remove impurities. The residual material was then dissolved in 0.05 M sodium carbonate by heating in a 100°C water bath for 10 minutes. After centrifugation to remove insoluble material, the solution was transferred to aluminium foil cups and dried overnight in an oven at 160°C. The dry residue was redissolved in warm 0.85 M KOH to obtain a 1 mg/mL solution of melanin. Melanin concentration was estimated by relating A_{400} values to known concentrations of the purified melanin. Variability in results was up to a maximum of 50% between replicate experiments and up to 20%

between duplicates in the same experiment. The absorption spectrum of the purified melanin was obtained by scanning in a Perkin-Elmer Lambda 3 spectrophotometer. Standard DOPA-melanin (Sigma), autooxidized catechol and autooxidized indole-melanin were used as authentic standards in this analysis.

3.2.4 Enzyme activity analysis

Cell-free extracts were obtained after sonication of the cells suspended in 0.5 M phosphate buffer, pH 6.8 using a Branson Sonic Power Sonifier Model 350. The cells were disrupted by four 20-s bursts of full power at 1-minute intervals. Cell debris was removed by centrifugation at 1935 x g for 10 minutes at 4°C and the protein content of the cell free extract was determined.

Phenol oxidase (E.C. 1.10.3.1.) activity was determined as described in the Worthington Enzyme Manual (1972). Enzyme activity was estimated by following the oxidation of catechol at A_{390} (Duckworth and Coleman, 1970) or L-tyrosine at A_{280}, at one minute intervals. In another procedure of Lerch and Ettlinger (1972), tyrosinase activity was measured using DL-DOPA as substrate and the formation of dopachrome was monitored by measuring the increase in absorbance at 475 nm using the Hitachi Model U-2000 double-beam spectophotometer. The molar extinction coefficient for the reaction at 475 nm is 3600 M^{-1} cm^{-1}.

Nitrogen-fixing activity was determined using 3 mL of cell suspension in a 10 mL Erlenmeyer flask which was sealed with a serum stopper and had 10% of the atmosphere replaced by acetylene. To determine the effect of oxygen on nitrogenase activity cells were grown in 48 mL medium in 160 mL serum bottles which were stoppered with serum stoppers. For estimating nitrogenase activity, the bottles were flushed with helium for 2 minutes, 10% of the atmosphere was replaced with acetylene, and the oxygen level in the head space of the bottle was adjusted by replacing helium with oxygen (eg. for 10% O_2, 11.2 mL of helium was replaced with the same amount of O_2). The flasks or bottles were incubated under the same conditions used for growth, for 1 hour, and ethylene produced was estimated by injecting 0.5 mL of the gas phase into a Hewlett Packard Gas Chromatograph (5890A) fitted with a Poropak R column. The specific activity of nitrogenase was expressed as micromoles ethylene produced (mg cell protein)$^{-1}$h^{-1}. There was up to 20% variability in results between replicate experiments.

Catalase and peroxidase activities were assayed as described previously (Page et al. 1988). Hydroxamate production was estimated as described previously (Page, 1987). Variability of up to 15% was observed in results obtained in replicate experiments.

3.2.5 Non-Denaturing Polyacrylamide Gel Electrophoresis

For the preparation of cell-free extracts, 200 mL culture was collected by centrifugation at 10,400 x g for 10 minutes. The cell pellet was resuspended in 1 mL of 0.05 M sodium phosphate buffer, pH 6.8. The cells were disrupted by sonication as described above. The lysate was treated for 30 minutes at room temperature with RNAase and DNAase (50 µg/mL final concentration of each) and then cleared of cell debris by centrifugation at 1935 x g for 20 minutes. The supernatant was saved. For further separation of membrane-associated proteins, the procedure of Schnaitman (1971) as described by Huyer et al. (1986) was followed. The above mentioned supernatant was spun at 17,700 x g in a 50Ti rotor in a Beckman ultracentrifuge at 4°C for 30 minutes. The supernatant was saved and the pellet resuspended in 0.05 M sodium phosphate buffer, pH 6.8. Triton X-100 was added to a final concentration of 2% and the suspension was sonicated in a Branson 1200 sonic bath for 2 minutes. After placing the suspension at 37°C for 20 minutes, the contents were centrifuged at 17,700 x g in a 50Ti rotor for 1 hour at 4°C. The pellet contained the membrane fraction of the cell extract.

Location of tyrosinase activity in non-denaturing discontinuous polyacrylamide gels was carried out using essentially the procedure of Laemmli (1970) except that no SDS was incorporated into the buffers. The SE 250 slab gel electrophoresis unit of Hoefer Scientific Instruments, San Fransisco, was used for the electrophoresis. The percentage of acrylamide in the resolving gel was 7%, 8%, 9% and 10% (w/v). The stacking gel acrylamide concentration was kept constant at 4%. The samples were run at 15 mA until the marker dye (bromophenol blue) had reached the bottom of the gel. The gel was stained for tyrosinase activity by soaking in a solution of 7 mM DL-DOPA in 0.05M sodium phosphate buffer, pH 6.8, for 4 hours or until the tyrosinase bands could be seen (Lerch and Ettlinger, 1972). The molecular weight markers used were Bovine serum albumin (66K, 132K, 198K, 264K), Jack bean urease (272K, 545K), Alcohol dehydrogenase (150K), and Ovalbumin (43K), where, K=1000 daltons. All these chemicals were obtained from Sigma Chemical Co. The position of the molecular weight markers was determined after staining the gel which had been previously stained with the enzyme substrate, with Coomassie blue as described in the Hoefer Scientific Instruments instruction manual. The size of the activity bands present in strain 184 were estimated by plotting the logarithmic value of the molecular weight standards versus the mobility of the protein in the presence of various concentrations of acrylamide. In another method as

described by Kingsbury and Masters (1970), the ratio of the relative mobilities (at the various acrylamide concentrations) for the standard proteins were estimated. Plots were then made of the ratios of the relative mobilities of the standard proteins against the logarithmic value of their molecular weights. Using these plots, the molecular weight of the activity bands in cell extracts of strain 184 were estimated and in most cases found to be similar to the values obtained by the method mentioned above.

3.3 RESULTS

3.3.1 Pigmentation of cells

Azotobacter chroococcum strain 184 cells became increasingly pigmented during growth in Burk's nitrogen-free medium containing 10 μM ferric citrate (Table 3.1). The colour of the cells changed from colourless to light brown after 72 hours, and to black at 96 hours, after which time there was no further detectable change.

The cell-associated pigment (Fig. 3.1; Chapter 4, Fig. 4.4) was extracted from the cells as described in Materials and Methods and identified as a melanin based on the following characteristics: solubility in hot 0.5 M NaOH and in 1 M Na_2CO_3, and insolubility in cold water, hot water, ethanol, chloroform, acetone, and cold 0.5 M NaOH. Upon addition of 0.1 M $FeCl_3$, a flocculent brown precipitate formed; the pigment was bleached by H_2O_2 (15%, w/v), and there was a decrease in intensity of the colour upon addition of glutathione (5%, w/v). The absorption spectrum of the purified pigment was characteristic of a melanin (Fig. 3.2).

When the organism was streaked out onto solid medium, it was observed that the colonies showed sectoring (Fig. 3.1). When the nonpigmented sectors were restreaked onto fresh plates, the same appearance of colonies was demonstrated, viz. non-pigmented sectors in black colonies. Thus the instability in melanin formation appeared to be reversible.

3.3.2 Effect of amino acids, charcoal, benzoic acid and light on melanin formation

Strain 184 pigmentation appeared normal when the cells were grown on nitrogen-free agar slants containing L-tyrosine, L-cysteine and L-tyrosine plus L-cysteine (Table 3.2). However, these amino acids did not promote melanin formation when ammonium acetate was the nitrogen source. Melanization also was unaffected by

Table 3.1 Melanin production and nitrogen fixing activity over a period of 5 days[a]

Incubation period hour	Protein[b]	Melanin[c]	Catechol[d]	Nitrogen fixation[e]
24	200	0.60	0.04	4.60
48	310	1.30	0.40	1.3
72	295	4.90	1.00	1.00
96	300	48.70	0.50	0.80
120	290	38.10	ND	0.00

a All values are means of at least two duplicates. Aliquots were withdrawn from the cultures (150 mL per 500 mL Erlenmeyer flask) at the appropriate time intervals.

b Expressed as microgram protein milliliter^{-1}.

c Expressed as milligram melanin (milligram of protein)$^{-1}$.

d Expressed as micromoles catechol (milligram of protein)$^{-1}$.

e Expressed as micromoles ethylene produced (milligram of protein)$^{-1}$ hour^{-1}.

ND Not determined.

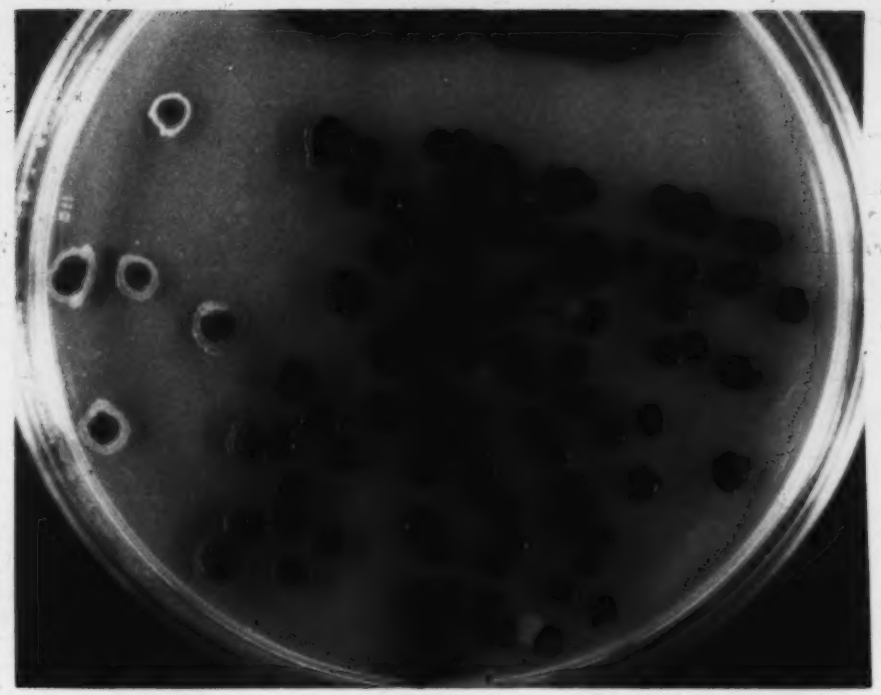

Fig. 3.1. Sectoring in colonies of strain 184. The organism was streaked onto solid Burk's medium supplemented with ammonium acetate (14 mM), sucrose (2%, w/v) and cuprous chloride (2.5 μM). Arrows indicate the position of nonpigmented sectors in black colonies of strain 184.

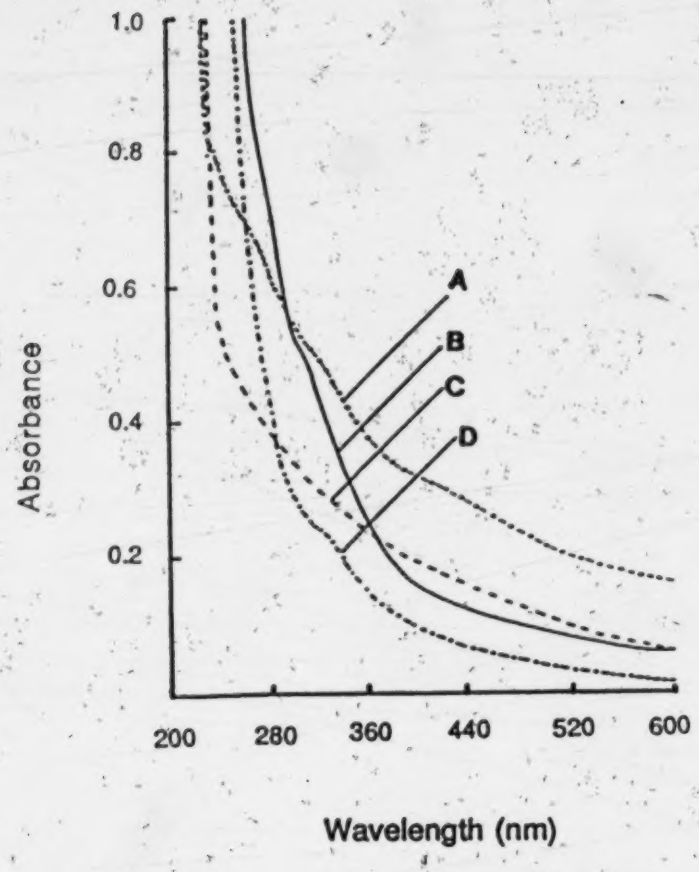

Fig. 3.2. Absorption spectra of autooxidized catechol-melanin (A), autooxidized indole melanin (B), standard DOPA-melanin (C), and purified strain 184 melanin (D).

Table 3.2 Effect of amino acids, copper and NH_4^+ on melanin formation[a]

Addition to growth medium	Melanization in:	
	Solid Medium[b]	Liquid Medium[c]
$-NH_4^+$		
None	+	34
CuCl (2.5 µM)	+++	38
L-tyrosine (0.1%)	+	16
L-tyrosine+ L-cysteine (0.1%)	+	18
L-cysteine (0.1%)	+	18
Charcoal (0.2%)[d]	ND	7
Benzoic acid (1 mM)	ND	4
$+NH_4^+$ (14 mM)[e]		
None	-	0
CuCl (2.5 µM)	+++	17
L-tyrosine (0.1%)	-	0
L-tyrosine + L-cysteine (0.1%)	-	0
L-cysteine (0.1%)	-	0

ND Not determined.

a All values are means of at least two duplicates.

b Melanization after 7 days incubation. +++ dark brown to black; + brown; - no brown colour.

c After 4 days incubation. Expressed as milligram melanin (milligram of protein)$^{-1}$.

d 14-60 mesh charcoal (Sigma).

e Ammonium was added as ammonium acetate.

incubation of slants in the light or in the dark (data not shown).

Quantitative studies using liquid growth media indicated that all the amino acids depressed melanin formation (Table 3.2). Melanization, was also suppressed by the presence of charcoal or benzoic acid in the medium. Significant stimulation of melanin formation (in the presence of NH_4^+) or intensification of melanin colour (in the presence of N_2) was caused by copper (see Chapter 4. Fig. 4.4).

3.3.3 Presence of catechol in culture supernatant fluids

After 72 hours incubation, the culture supernatant fluid of nitrogen-fixing strain 184 contained a compound which absorbed strongly at 272 nm (Fig 3.3) and was reactive in the Barnum assay for o-dihydric phenols. Its migration pattern on silica gel using the solvents systems described in the Methods, was identical to that of authentic catechol. The changes in colour on addition of iron as ferric chloride accompanied with pH changes were characteristic of catechol viz., blue (pH 4.8), purple (pH 6.5), wine red (pH 7.1), red (pH 7.6).

The red colour developed in the Barnum assay also showed an identical absorption maximum to that of standard catechol. Gas chromatographic-mass spectrometric analysis of the purified compound clearly identified the compound as catechol (Fig. 3.4) showing a strong molecular ion at m/z= 110. Resorcinol (1,3-benzenediol) and hydroquinone (p-dihydroxybenzene), which had the same molecular weight as catechol, did not react in the Barnum assay.

Catechol was produced under a range of aeration conditions imposed by shaking different volumes of media at 250 rpm (Table 3.3). Catechols released into the culture supernatant fluid demonstrated an inverse relationship with melanization according to increased aeration. Furthermore, it was observed that as the cells melanized, the catechol disappeared from the culture supernatant fluids (Table 3.1). These data, combined with the spectral data (Fig. 3.2) suggested that the strain 184 melanin was a catechol-melanin.

Melanization was absent when the cells were grown under static, very low aeration or microaerophilic growth conditions. Melanization at low aeration conditions (shaking at 150 rpm) was enhanced by the presence of iron in the medium. With 10 µM ferric citrate there was no melanization after 96 hours, but with 50 and 100 µM ferric citrate the cells had melanized to a light brown colour. Vigorously aerated cultures, on the other hand, melanized by 72 hours at all iron levels.

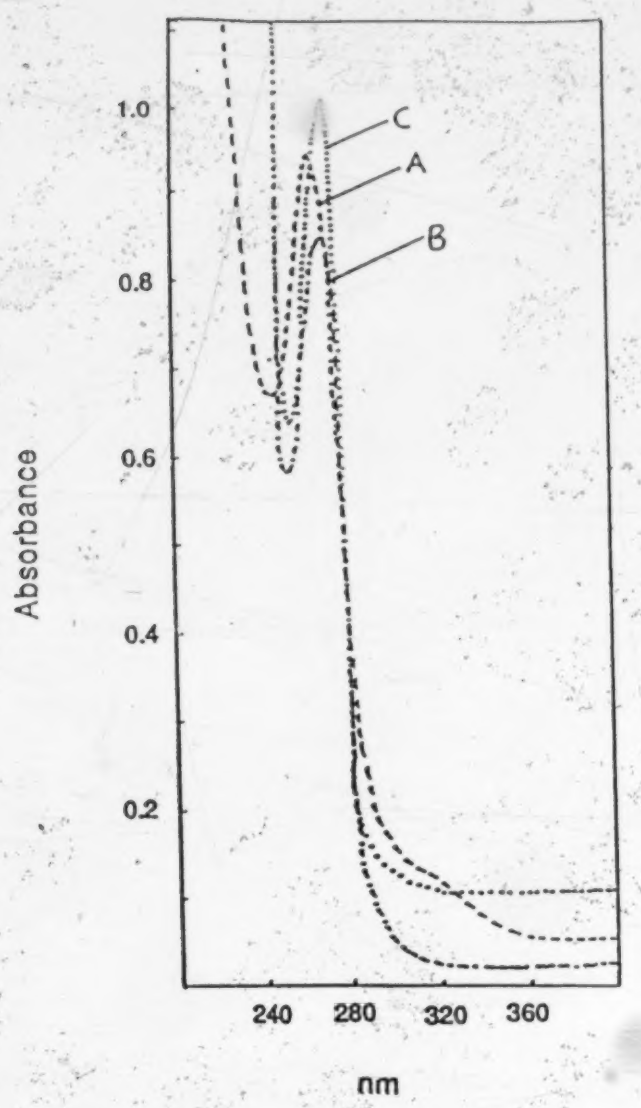

Fig. 3.3. UV scan of cell-free culture supernatant fluid of *Azotobacter chroococcum* strain 184 grown in Burk's medium under nitrogen-fixing conditions (A), ethyl acetate extract of culture supernatant (B), and catechol purified from culture supernatant of strain 184 which was redissolved in methanol:chloroform (5:95, v/v) (C).

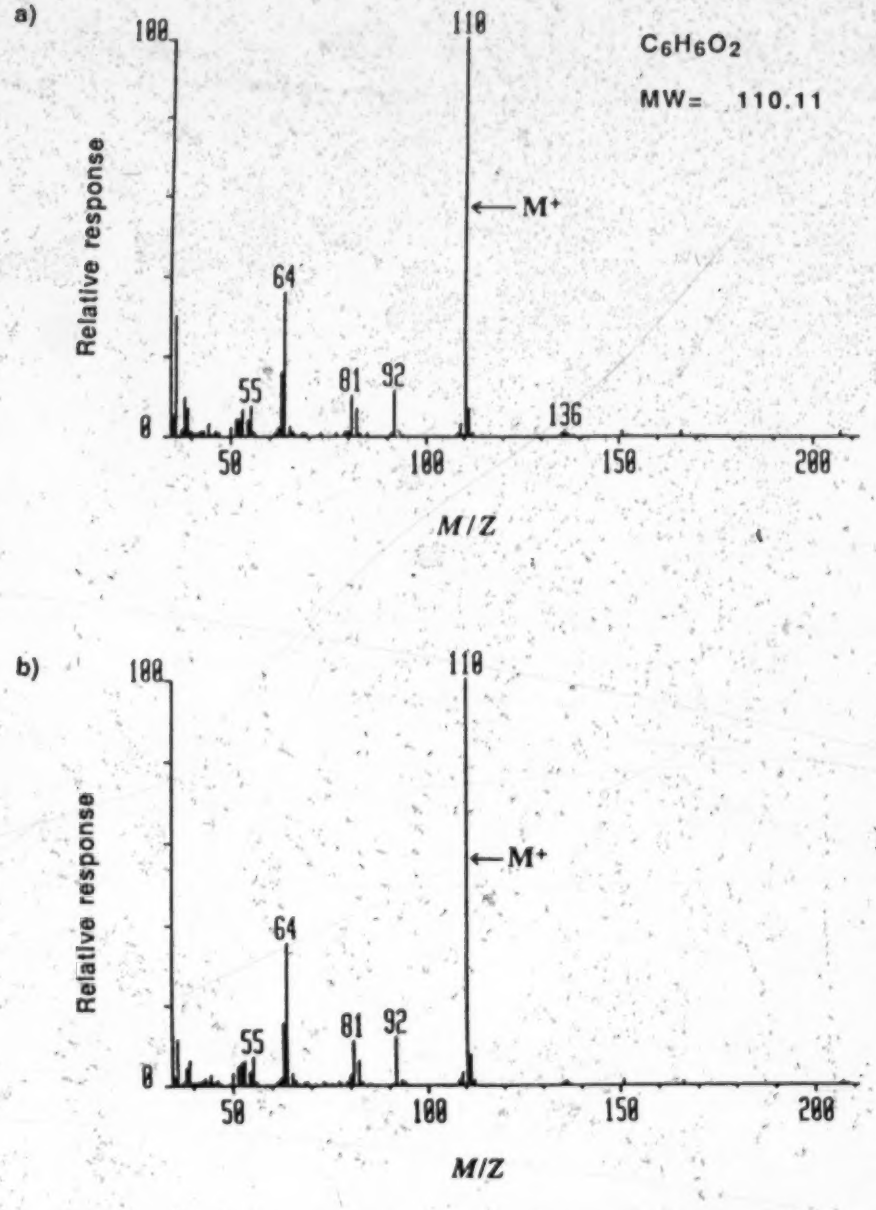

Fig. 3.4. Mass spectrometric analysis of a) pure catechol and b) compound purified from culture supernatant of *Azotobacter chroococcum* strain 184. The compound present in the culture supernatant of strain 184 had a retention time (4.43 minutes) identical to that of pure catechol when analyzed by gas chromatographic techniques. The compounds were analyzed on a 30-m DB-1 capillary column with helium as carrier gas at a flow rate of 45 cms^{-1} and a holding temperature of 90°C as described in Materials and Methods.

Table 3.3 Effect of aeration on hydroxamate, catechol and melanin formation by nitrogen-fixing cells[a]

% culture volume per flask	Protein[b]	Hydroxamate[c]	Catechol[d]	Melanin[e]
10	320	3.00	0.20	10.30
20	300	1.10	0.80	18.10
30	270	0.15	1.00	32.00
40	220	*f*	3.40	4.50
50	220	-	3.80	1.80
60	180	-	ND	1.70

a All values are means of at least two duplicates.

b Expressed as microgram protein milliliter^{-1} after 40 hours of growth.

c Expressed as microgram hydroxamate-nitrogen (milligram of protein)$^{-1}$ after 40 hours of growth.

d Expressed as micromoles catechol (milligram of protein)$^{-1}$ after 72 hours of growth.

e Expressed as milligram melanin (milligram of protein)$^{-1}$ after 96 hours of growth.

f Not detectable.

ND Not determined.

3.3.4 Oxygen effect on nitrogenase activity and hydroxamate production

Strain 184 appeared to have maximum nitrogenase activity at oxygen concentrations lower than atmospheric concentrations. 10% O_2 (initial head space concentration) supported the highest nitrogenase activity. The activity at 20% O_2 and 5% O_2 was 66% and 76%, respectively, of the activity at 10% O_2 after 18 hours of growth.

Further, when strain 184 was grown under microaerophilic or static conditions, it did not produce the hydroxamate siderophore reported earlier (Page, 1987). Similarly, when grown at varying aeration rates, hydroxamate production was not detectable at lower aeration rates (Table 3.3). The iron content of the cells varied from 1.27-1.41 μg Fe^{3+} (mg protein)$^{-1}$ at the higher aeration rates to 0.77-0.98 μg Fe^{3+} (mg protein)$^{-1}$ at the lower rates of aeration. Cells from cultures grown at higher rates of aeration contained 70-100% of the added iron after 40 hours, whereas, cells grown at the lower aeration rates had 30-50% of the added iron. Hydroxamate production was probably repressed at low aeration due to the presence of repressive levels of iron which remained in the growth medium due to low iron utilization. These levels of iron per protein were much higher than the iron-limited levels of 0.36-0.59 μg Fe^{3+} (mg protein)$^{-1}$ reported previously (Page, 1987), although cultures at higher aeration were apparently becoming externally iron-limited.

3.3.5 NH_4^+ effect on melanization and catechol formation

As shown in Table 3.2, NH_4^+ appeared to repress melanization. Levels of NH_4^+ that completely inhibited nitrogenase activity (14 mM) also prevented melanization and catechol production (Table 3.4). Lower levels of NH_4^+ which partially inhibited nitrogenase activity also delayed melanization and depressed catechol production. Levels of NH_4^+ which did not depress nitrogenase activity also were without effect on catechol formation and melanization (Table 3.4). When NH_4^+ (14 mM) was added at various stages of growth under nitrogen-fixing conditions it was observed that catechol production was suppressed when ammonium was added during the lag phase (0 hour to 10 hour) and during the exponential phase of growth (10 hour to 28 hour). However, when NH_4^+ was added during the stationary phase (after 28 hour), catechol had already been produced and melanization occurred, although to a lesser extent (data not shown).

KNO_3 (14 mM) and urea (0.1%, w/v) also repressed catechol formation and therefore melanization. The amino acids, L-tyrosine, L-tryptophan, L-glutamine supported catechol formation (Table 3.5). In the presence of DL-histidine a lower level

Table 3.4 Effect of NH_4^+ on catechol and melanin production and nitrogenase activity[a]

Incubation conditions	Incubation period	Catechol[b]	Nitrogenase[c]	Time of initial melanization and final intensity[d]
Nitrogen-fixing	24	0.04	2.67	96 h (+++)
	48	0.97	0.96	
2 mM NH_4^+[e]	72	1.00	*f*	
	24	0.04	2.87	96 h (+++)
	48	0.07	0.99	
4 mM NH_4^+	72	1.1	-	
	24	0.04	0.86	96 h (++)
	48	0.42	0.86	
8 mM NH_4^+	72	0.85	-	
	24	0.03	0.90	/ d (++)
	48	0.09	0.95	
14 mM NH_4^+	72	0.1	-	
	24	0.02	-	absent
	48	0.04	-	
	72	0.02	-	

a All values are means of at least two duplicates.

b Expressed as micromoles catechol (milligram of protein)$^{-1}$.

c Expressed as micromoles ethylene produced (milligram of protein)$^{-1}$ hour^{-1}.

d +++ dark brown to black; ++ brown.

e NH_4^+ was added as ammonium acetate.

f Not detectable.

Table 3.5 Effect of oxygen radical scavengers and amino acids on catechol formation[a]

Addition(s) to growth medium	Protein[b]	Catechol[c]
-NH_4^+[d] (with oxygen radical scavengers)		
None	270	1.00
Mannitol (10 mM)	270	1.10
DL-Histidine (10 mM)	320	0.30
Charcoal (0.2%)[e]	250	0.08
Catalase (70 units/mL)	250	1.00
-NH_4^+[f] (with amino acids)		
None	250	0.15
L-Tyrosine (10 mM)	270	0.60
L-Tryptophan (10 mM)	340	0.70
L-Glutamine (10 mM)	280	0.90
Peptone (0.1%)	290	2.40
+14 mM NH_4^+[g]		
+ MSX (75 μM)[h]	240	0.40

a All values are means of at least two duplicate determinations.

b Expressed as microgram protein milliliter^{-1}.

c Expressed as micromoles catechol (milligram of protein)$^{-1}$.

d After 72 hours of incubation.

e 14/60 mesh charcoal (Sigma).

f After 43 hours of incubation.

g After 72 hours of incubation.

h Methioinine sulfoximine (Sigma).

of catechol production was observed, although growth was enhanced. Histidine-grown cells were nitrogen-fixing as observed by the acetylene reduction assay (data not shown). In the presence of peptone, the production of catechol was exceptionally high even at 43 hours of growth (Table 3.5).

In the presence of NH_4^+, derepression of catechol formation was observed when methionine sulfoximine (75 μM) was incorporated into the growth medium (Table 3.5). The level of catechol formation did not, however, reach the same level as seen in nitrogen-fixing cells.

The culture fluids from NH_4^+-grown cells, after periods of incubation from 1 to 3 days, did not contain any inhibitory compound which would inhibit catechol and melanin formation when added to nitrogen-fixing cells. This suggested that NH_4^+-repression occurred as a result of an internal change and not through some excreted product.

In earlier studies, polyphenol oxidase activity was observed only in cell-free extracts prepared from 72-hour nitrogen-fixing cells but not from NH_4^+-grown cells. Enzyme activity was not observed in the cell free extracts prepared from 24-hour and 48-hour N_2-fixing cultures. However, when approximately 1 μmole catechol (mg protein)$^{-1}$ was observed in the culture supernatant fluid, polyphenol oxidase activity (using catechol as a substrate) was detectable in the cells [0.0052 Δ A_{390} min^{-1}(mg protein)$^{-1}$] which suggested initially, that catechol was involved in the induction of this enzyme. However, when more concentrated cell extracts were prepared and analyzed for enzyme activity using DL-DOPA as substrate rather than catechol, both NH_4^+-grown [7.58 nmoles dopachrome formed min^{-1} (mg protein)$^{-1}$] and nitrogen-fixing cells [4.9 nmoles dopachrome min^{-1} (mg protein)$^{-1}$] demonstrated polyphenol oxidase activity even in 24 hour old extracts.

3.3.6 Effect of free radical scavengers on catechol formation

As seen in Table 3.5, among the free radical scavengers, histidine and charcoal appeared to have a repressive effect on catechol formation. In the presence of histidine, the growth of the organism was enhanced relative to that in medium with no supplements and the organism was still growing under nitrogen-fixing conditions as demonstrated by a positive reaction in the acetylene-reduction assay (5.8 μmole of ethylene produced per mg protein per hour). Catalase and mannitol had no effect on catechol formation (Table 3.5).

3.3.7 Melanin formation and nitrogenase activity

Melanin formation and nitrogenase activity were monitored over a period of 5 days (Table 3.1). It was observed that maximum melanization occurred after 4 days of growth, whereas, nitrogenase activity was at its peak in the early stages of growth and was very low after 3 days (Table 3.1 and Table 3.4). Thus, there was no correlation between nitrogenase activity and melanin formation which suggested that melanin per se was not playing a protective role for nitrogenase activity.

3.3.8 Effect of copper on catechol formation and melanization

When strain 184 was grown in media containing copper (Cu^{2+} or Cu^{1+}), melanization was accelerated so that peak production in liquid medium occurred after 48 hours. Copper also stimulated melanization of cells grown in the presence of NH_4^+ (Table 3.2) and, therefore appeared to partially reverse the inhibitory effects of NH_4^+.

Another interesting observation was that cells grown in the presence of ammonium and copper did not seem to produce detectable catechol in the culture supernatants. An orange colour developed in the Barnum assay of culture supernatants, which had an absorption maximum of 489 nm after 2 minutes and 486 nm after 30 minutes reaction time. This colour was unstable and disappeared rapidly. In the Barnum assay, the λmax of catechol was 503 nm both after 2 minutes and 30 minutes, although the intensity of colour was optimal after 30 minutes. DL-DOPA had a λmax of 499 nm after 2 minutes reaction time which changed to 493 after 30 minute reaction time.

3.3.9 Non-denaturing polyacrylamide gel electrophoresis

As seen in Fig. 3.5 at least two common tyrosinase activity bands were seen in cell extracts from both nitrogen-fixing and ammonium-grown cells. By comparing the relative mobility of the tyrosinase and protein standards on non-denaturing gels with concentrations of 7-10% acrylamide the molecular weight of the tyrosinase was estimated to be 111K (K=1000 daltons) and 127K (Fig. 3.6). In ammonium-grown cells the major activity band corresponded to 111K with minor bands of 127K and 151K, whereas, in the nitrogen-fixing cells, the major band in the both the soluble fraction and the membrane fraction corresponded to a molecular weight of 127K and the minor band corresponded to 111K. The membrane fraction of ammonium-grown cells had only a single band corresponding to 111K.

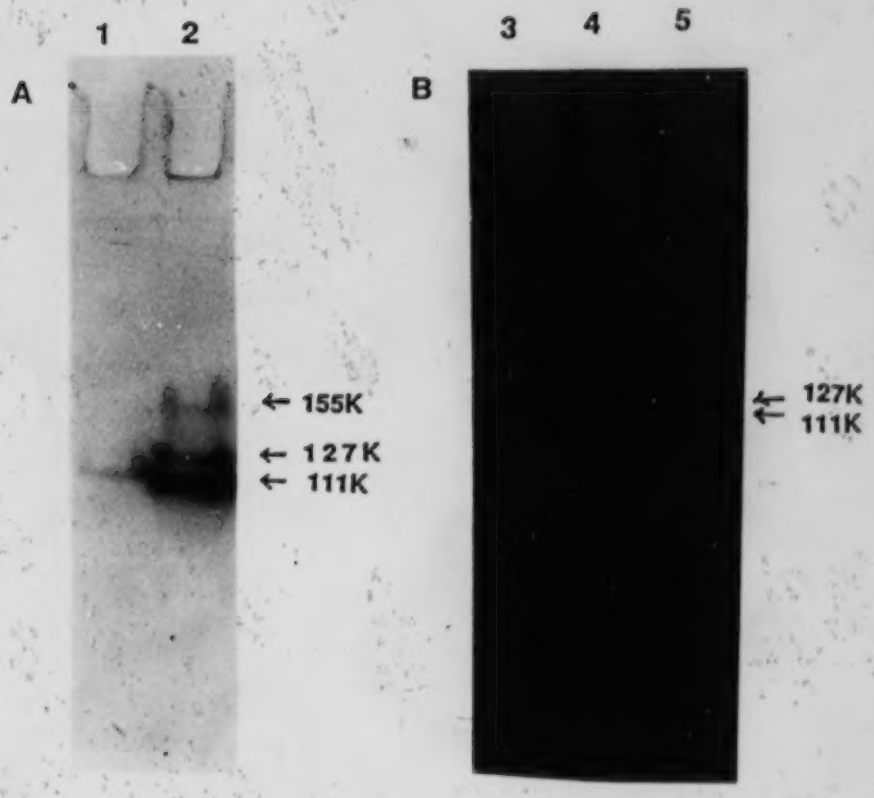

Fig. 3.5. Non-denaturing gel electrophoresis of cell extracts of strain 184 grown with and without ammonium. The gel was stained for tyrosinase activity bands by soaking it in a solution of DL-DOPA (7 mM) in 0.05 M sodium phosphate buffer, pH 6.8, for 4 hours. A) Arrows indicate the activity bands in membrane fraction of ammonium-grown cells (Lane 1), and the soluble cytoplasmic fraction (Lane 2) of the same. B) Arrows indicate the activity bands in the membrane fraction of nitrogen-fixing cells (Lane 3,4) and the soluble cytoplasmic fraction (Lane 5). (The enzyme activity bands in the nitrogen-fixing cells are not very clear, the negative used for the prints shows more defined bands).

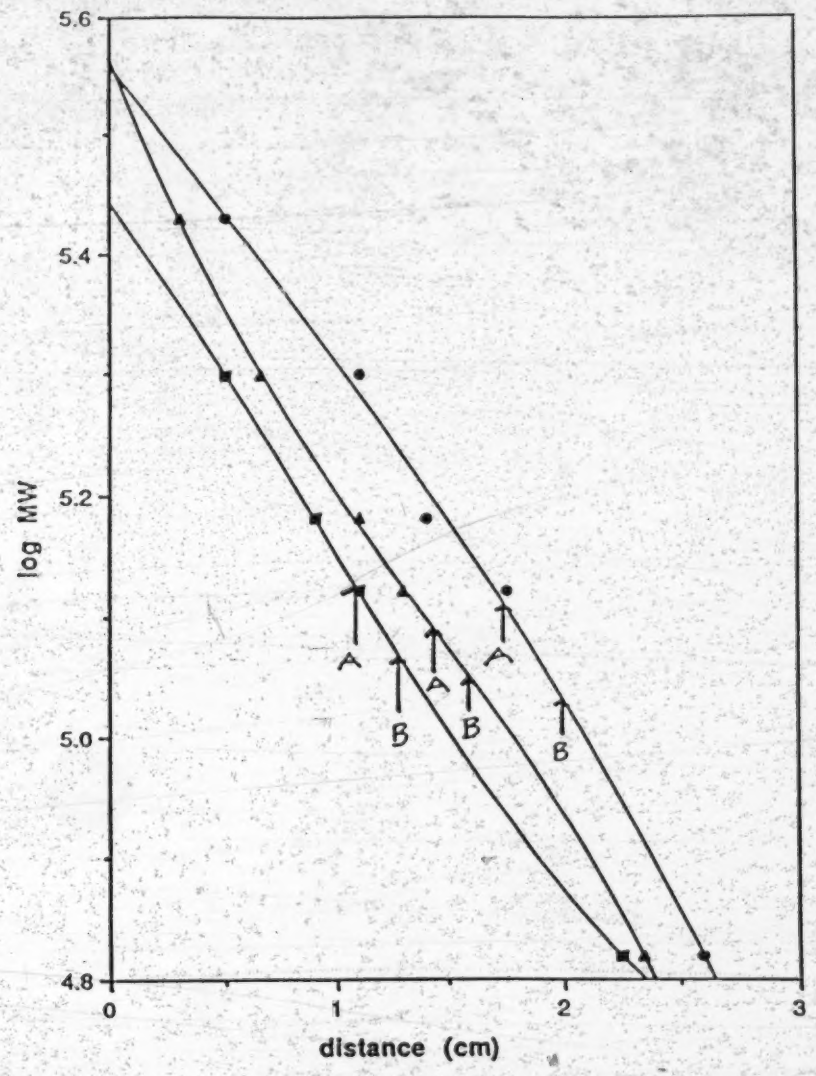

Fig. 3.6 Mobility of protein standards at 8% (●), 9% (▲), and 10% (■) polyacrylamide versus the logarithm of the molecular weight of the protein standards on analysis by non-denaturing polyacrylamide gel electrophoresis. Polynomial curve fits are shown. Standard proteins: Bovine serum albumin (66K, 132K, 198K, and 264K daltons); Alcohol dehydrogenase (150K daltons), and Jack Bean Urease (272K and 545K daltons). Arrows indicate positions of the 127K dalton isoenzyme (A), and the 111K dalton isoenzyme (B), which are present in the cell extracts of nitrogen-fixing and ammonium-grown strain 184.

3.3.10 Effect of copper on catalase and peroxidase levels in strain 184

In the presence of copper, strain 184 did not contain detectable catalase or peroxidase activities. However, copper did not inhibit aerobic growth under these conditions as indicated by similar protein values. The addition of 15 μM copper did not affect the *in vitro* activity of active catalase or peroxidase in cell free extracts. Thus it would appear that in the presence of copper, strain 184 can still grow substantially even in the absence of the enzymes that normally would be required for aerobic growth and would normally protect the cell from toxic oxygen products.

3.4 DISCUSSION

When *Azotobacter chroococcum* strain 184 is grown under nitrogen-fixing conditions and relatively high aeration, it produces a cell-associated melanin in the latter stages of its growth cycle.

Catechol, which is produced by the organism disappears upon melanization suggesting that the melanin is derived from catechol and that the catechol may be both an inducer and a substrate for melanization. Bortels and Henkel (1968) report that the dark pigment formed by *Azotobacter chroococcum* is not a melanin but is related to the gray humic acids. However, in strain 184, the chemical characteristics of the melanin and the presence of polyphenol oxidase activity in cell-free extracts conclusively identifies the pigment as melanin. Catechol-melanins have been observed previously in plant seeds and in the spores of *Ustilago maydis* (Piatelli et al. 1963; Nicolaus, 1968). The amino acids, L-tyrosine and L-cysteine did not enhance melanin formation in strain 184, unlike the melanization of *Vibrio cholerae* (Ivins and Holmes, 1980) and *Azospirillum brasilense* (Sadasivan and Neyra, 1987), but in fact, decreased its formation to a certain extent. Based on these results the Na^+-dependent strain of *Azotobacter chroococcum* appears to produce an allomelanin rather than a eumelanin or phaeomelanin.

Maximum pigmentation of strain 184 occurred at relatively high aeration and pigment formation was suppressed by O_2 limitation. In another microaerophile, *Azospirillum brasilense*, carotenoid pigment formation was observed only under aerobic conditions (Nur et al. 1981). Melanin formation was not observed in cells grown under static growth conditions or when grown microaerobically. Thus melanin formation

appears to be necessary, or as a consequence of high aeration rates. Ivins and Holmes (1980) similarly report melanin production by *Vibrio cholerae* Htx-3 when grown under higher aeration and not when grown under static or anaerobic growth conditions. These results could be simply due to the presence or absence of O_2 which participates directly in the enzymatic oxidation of polyphenols (Mason, 1957).

There appears to be genetic instability in the expression of the polyphenol oxidase in strain 184. The same sectoring, viz. white sectors in otherwise black colonies, was repeated when non-pigmented sectors in pigmented colonies were restreaked onto fresh medium of the same composition. Thus the genetic instability seemed to be reversible. In *Streptomyces* species, several phenotypic traits have been reported to be unstable (Freeman et al. 1977; Shaw and Piwowarski, 1977; Freeman and Hopwood, 1978; Hütter and Eckhardt, 1988). The basic instability in *Streptomyces* is known to preferentially affect characters which are developmentally regulated (Leblond et al. 1990). Among these characters are, aerial mycelium formation, spore production, antibiotic resistance and/or production, and the formation of extracellular enzymes, among which, one is tyrosinase (Leblond et al. 1990). Leblond et al. (1990) reported that many organisms which display differentiation or a complex developmental cycle also exhibit large genomic rearrangements such as site-specific recombinations and deletions to create genes or expression frames as has been seen in *Bacillus subtilis* (Stragier et al. 1989) and in *Anabaena* species (Golden et al. 1988). Among the possible causes of genetic instability in *Streptomyces* are plasmid loss (Leblond et al. 1990) and extensive chromosomal deletions often accompanied by intense DNA amplifications (Birch et al. 1989). Another explanation for unstable traits is the integration-excision cycle of transposon-like sequences, resulting in reversible instability of streptomycin resistance in *Streptomyces griseus* and *Streptomyces bikiniensis* (Kirby and Lewis (1981). *Azotobacter* also displays differentiation as they demonstrate encystment in media containing n-butanol (Socolofsky and Wyss, 1961), or having mineral deficiencies (Layne and Johnson, 1964) or following active growth phase, by aging cells. In strain 184 the production of melanin was evident only after active growth since maximal pigmentation was observed at 96 h of growth. The similarities in the genetic instability in melanin formation between this organism and *Streptomyces* species is interesting and a genetic analysis of this phenotype should provide an explanation for this instability.

In the presence of charcoal, a free radical trap and an adsorbent of toxic O_2 intermediates (Hofmann et al. 1983), strain 184 cells do not melanize. Hoffman et al.

(1983) used charcoal in laboratory media to increase the aerotolerance of microaerophilic organisms such as *Legionella* spp. Similarly, benzoic acid, which can act as a scavenger of hydroxyl radicals (Gutteridge, 1987), also had a suppressive effect on melanization. These results suggest that there may be an inverse relationship between catechol formation and /or melanization and the presence of toxic oxygen species in strain 184. In the presence of free radical scavengers such as histidine and charcoal, catechol formation was repressed. In the presence of charcoal, catechol fromation was not detectable. Charcoal can destroy hydrogen peroxide and superoxides, and it is also a weak reducing agent which can trap free radicals and thereby prevent the formation of superoxide and hydrogen peroxide (Hofman et al. 1983). It is also possible that charcoal could have adsorbed catechol present in the culture supernatants due to its high adsorptive capacity (Hoffman et al. 1983). However, since in an earlier study, charcoal enhanced the recovery of cells of strain 184 during plating on solid media (Chapter 2; Page et al. 1988) this latter possibility was not investigated further. Histidine quenches hydroxyl radicals and singlet oxygen (Hoffman et al. 1979). In the presence of histidine, growth was also enhanced and the organisms were still growing under nitrogen-fixing conditions, which indicated that the amino acid did not repress nitrogenase. Badawy (1977) in a study on the effect of amino acids on the growth and morphology of *Azotobacter chroococcum* demonstrated that L-histidine (0.002 M) stimulated growth during the exponential phase. In a study by Brawn and Fridovich (1981), ColEI DNA suffered strand scission when exposed to superoxides. Hydroxyl radical scavengers such as mannitol, benzoate and histidine prevented such strand scission. Thus, the hydroxyl radical scavenging ability of histidine could be providing a protective role for the organism from toxic oxygen radicals thereby decreasing the requirement for catechol formation. This further supports the hypothesis for the protective role of catechol and/or melanization for nitrogen-fixing cells.

Copper accelerated melanin formation and also increased the intensity of the pigment colour in strain 184. This ion is an integral part of polyphenol oxidase and a known stimulator of enzyme activity (Mason, 1957). Interestingly, copper eliminated the catalase and peroxidase activities of strain 184 but did not prevent aerobic growth of the cells. These results indicate that an alternative mode of protection from toxic oxygen species must exist in these cells.

The presence of a Barnum-reactive compound in copper-grown cells with and without ammonium indicates the presence of an o-dihydric phenol, which was, however, not catechol. Therefore, the melanin formed would not be a catechol-melanin. It is

possible that it is a DOPA-melanin and the organism could be using internal tyrosine as a substrate for the polyphenol oxidase. Furthermore, since no melanin formation was observed when the cells were grown with ammonium alone, it indicated the presence of an inactive polyphenol oxidase which was activated when trace amounts of copper was added to the growth medium. It is possible that the trace amounts of copper in the normal growth medium were diluted relative to cell protein produced by the ammonium-grown cells, whereas, nitrogen-fixing cells which produce less cell mass in the same time period contain more copper/protein for enzyme activity. In earlier experiments no polyphenol oxidase activity was detectable in cell-free extracts prepared from ammonium-grown cells. However, on preparation of more concentrated cell-free extracts and using DOPA as a substrate for the assay instead of catechol, polyphenol oxidase activity could be demonstrated in both nitrogen-fixing and ammonium-grown cells. It is possible that in the earlier experiments the polyphenol oxidase in ammonium-grown cells was present, for the most part, in an inactive form and the active enzyme was simply too dilute to be detected. The more concentrated extracts therefore contained a higher percentage of active enzyme. Polyphenol oxidase activity bands were also demonstrated both in cell-free extracts of nitrogen-fixing and ammonium-grown cells after native gel electrophoresis.

Multiple polyphenol oxidase activity bands were observed in the cell extracts of strain 184. The enzymes have an apparent molecular weights of 111K and 127K. A minor band of molecular weight 151K was also seen in ammonium-grown cells. Strangely, the enzyme produced by *Azotobacter chroococcum* strain 184 appears to be closer in molecular weight to the tyrosinase from mushroom (128K) (Jolley et al. 1969) than to the isoenzymes from *Vibrio tyrosinaticus* (41K and 38.5K) (Pomerantz and Murthy, 1974), or those from *Streptomyces antibioticus* and *Streptomyces glaucescens* which have molecular weights of 29.5K (Bernan et al. 1985) and 29.1K (Lerch and Ettlinger, 1972), respectively. Tyrosinases from mouse and hamster melanoma have molecular weights of 55K and 70K respectively and that from *Neurospora crassa*, 33K. Four isoenzymes from mushroom tyrosinase were identified by electrophoresis, of which three had similar sedimentation coefficients. The enzyme was found to be a tetramer with each monomer having a molecular weight of 32,000 daltons (Duckworth and Coleman, 1970). It is possible that the two forms of the tyrosinase found in strain 184 are isoenzymes. Differences in charge and therefore mobility of the two forms would cause the enzymes to band at different points in the gel. The major activity band in ammonium-

grown cells was the 111K isoenzyme whereas in nitrogen-fixing cells it was the 127K isoenzyme although both enzyme bands were present in both extracts. Further information about the enzyme can be obtained by analytical ultracentrifugation and a study of subunit structure when the enzyme is purified.

Azotobacters normally display a high respiratory rate under nitrogen-fixing conditions to scavenge O_2 and create a suitable reducing environment for nitrogenase (Robson and Postgate, 1980). The Na^+-dependent *A. chroococcum* also appear to use respiratory protection of their nitrogenase when grown aerobically (Page et al. 1988) and nitrogen-fixing cells would therefore be expected to produce greater amounts of toxic O_2 species than NH_4^+-grown cells. The autooxidation of reduced flavins, the oxidation of reduced ferredoxins and the reactions of xanthine oxidase, aldehyde oxidase and dihydroorotic dehydrogensase and several flavin dehydrogenases are all involved in the generation of superoxides (Fridovich, 1975). Strain 184 possesses an active superoxide dismutase (Chapter 2; Page et al. 1988), but the product of this enzymatic reaction would be H_2O_2. The low levels of catalase activity seen in this strain does not increase in response to increased aeration (Chapter 2; Page et al. 1988). Whatever the oxygen protection system of strain 184 is, it must be relatively inefficient compared to the enzymatic protection present in other azotobacters, because the addition of small amounts of H_2O_2 is very toxic to the Na^+-dependent strain (Page et al. 1988).

The Na^+-dependent strains of *A. chroococcum* possibly have developed an oxygen protection system based on catechol formation and melanization which is especially important for aerobic growth under nitrogen-fixing conditions.

The superoxide-dependent formation of hydroxyl radicals requires the presence of transition metal ions and it has been found that iron and copper are the most effective (Halliwell, 1978). Both ions accelerated melanization in strain 184. Molecules which bind iron can prevent it from participating in the Fenton reaction and thereby protect a cell from the damaging effects of the hydroxyl radical. Such a role has been suggested for hydroxyl radical scavengers mannitol, glucose and thiourea, which bind iron, although weakly (Gutteridge, 1984). Imlay and Linn (1988) attribute a major portion of the toxicity in *E. coli* to DNA damage mediated by a Fenton reaction where hydrogen peroxide reacts with DNA-bound iron to generate hydroxyl radicals. Gutteridge (1987) reports the higher efficiency of hydroxyl radical scavengers with higher iron-binding affinities than the ligand so that the scavenger can protect the ligand by transferring the damage to itself. Catechol is such an iron-binding molecule and could itself be a target

for the hydroxyl radical. In a model chemical system, the catechol, Tiron, (1,2-dihydroxybenzene-3,5-disulfonic acid), is aerobically oxidized to the o-semiquinone radical by superoxides (Miller and Rapp, 1973). Semiquinone radicals are also intermediates in the formation of catechol melanin (Bell and Wheeler, 1986). Catechols, such as Tiron, can react directly with reduced flavin-O_2 compounds resulting in the oxidation of the catechol, and metalloflavoproteins on reaction with oxygen also result in the oxidation of Tiron (Miller and Rapp, 1973). Hydroxylation of aromatic compounds has been used as an *in vitro* assay for the production of the hydroxyl radical from superoxides (Richmond et al. 1981). Higher levels of iron result in higher levels of hydroxylation (Halliwell, 1978). Salicylic acid in *Mycobacterium* species and dihydroxybenzoic acids in *Enterobacter aerogenes* have been reported to accumulate in culture media of iron-deficient organisms (Ratledge, 1964). Salicylic acid and phenol were hydroxylated to diphenols by the hydroxyl radical in *in vitro* hydroxylation studies (Halliwell, 1978; Richmond et al. 1981). Thus catechol formation could be mediated by hydroxy radicals and the catechol so formed could be oxidized to further protect the cell from superoxide-mediated oxidative damage.

Due to the ability of melanins to bind cations like iron, melanins may protect cell membranes from damage by hydroxyl radicals generated via the Fenton reaction. Pilas et al. (1988) report a decrease in hydroxyl radical formation when ferrous ion is bound to melanin at low iron concentrations. Halliwell and Gutteridge (1986) emphasize the importance of metal sequestration in minimizing iron-dependent radical reactions, which are implicated in the pathogenesis of several diseases such as atherosclerosis, cancer and arthritis. Conversely, Aver'yanov et al. (1987) report an increased sensitivity of the mutant spores of *Pyricularia oryzae* which lacked melanin, to the hydroxyl radical and the superoxide radical. Melanins also have the ability to oxidize 80% of the $O_2^{\cdot}$ to O_2 and reduce 20% of supplied $O_2^{\cdot}$ to H_2O_2, thus demonstrating a pseudo-superoxide dismutase-peroxidase activity (Korytowski et al. 1986). In a series of experiments Sichel and coworkers demonstrated that melanin reduced lipoperoxidation in pigmented tissue (Scalia et al. 1990), and that there was an inverse relationship between SOD levels and melanin content in various tissues (Geremia et al. 1984; Sichel et al. 1987; Sichel, 1988; Geremia et al. 1989). Since hydroxyl radicals have ben reported to cause lipid peroxidation (Halliwell and Gutteridge, 1988), and free radicals also cause protein degradation (Wolff et al. 1986), the cell-associated melanin produced by strain 184 must be playing a role in protecting the cell membrane from oxygen radical-mediated damage.

The ability of melanin to remove harmful oxygen species without the formation of significant levels of H_2O_2 would be advantageous to the cell which lacks catalase and has low peroxidase activity.

The hydroxamate deferrioxamine inhibits hydroxyl radical formation and lipid peroxidation by the iron-catalysed Fenton reaction by chelating iron and rendering it less reactive (Gutteridge, 1984). Strain 184 produces a hydroxamate-type siderophore, which appears to function in iron assimilation but its regulation is unusual (Page, 1987). This hydroxamate is formed in the presence of iron concentrations that would normally repress the formation of a siderophore (Page, 1987). As shown in this study, the hydroxamate also appears more important to aerobically grown cells than microaerobically grown cells, although both must grow by respiratory metabolism. It is possible that the iron binding ability of the siderophore and thereby its ability to diminish the reactivity of free iron in the Fenton reaction could further protect the cell from oxidative damage.

As noted previously, catechol formation and melanization appears most important to nitrogen-fixing cells, possibly as a consequence of respiratory protection of nitrogenase and the increased number of iron-containing redox enzymes and potential sites for oxygen inactivation (Robson and Postgate, 1980). In *Azospirillum brasilense*, another microaerophilic organism, carotenoids have been suggested to play a role in protecting the organism from oxidative damage (Nur et al. 1981) due to the ability of this pigment to quench singlet oxygen and oxygen radicals (Krinsky, 1979). When nitrogenase is repressed by NH_4^+, the production of catechol and melanization also is repressed. Interestingly, carotenogenesis was also repressed by NH_4Cl in *A. brasilense* (Nur et al. 1981). Nitrate and urea also repressed catechol formation in strain 184. Nitrate is known to repress nitrogenase in azotobacters (Drozd et al. 1972). Catechol was still formed in the presence of amino acids, such as glutamine, and in the presence of peptone (fixed nitrogen compounds), conditions under which nitrogenase is still active in *Azotobacter* species and the organism is more oxygen stressed than when growing with ammonium as nitrogen source (Drozd et al. 1972). Furthermore, in the presence of the glutamine analog, methionine sulfoximine, catechol formation was derepressed in the presence of ammonium. Methionine sulfoximine, an inhibitor of glutamine synthetase, has been shown to derepress nitrogenase activity in *Azotobacter chroococcum* (Cejudo and Paneque, 1986). Thus the production of catechol appears to be regulated in a manner similar to nitrogenase. Since *nifA* regulates melanin formation in *Rhizobium*

leguminosarum biovar *phaseoli* (Hawkins and Johnston, 1988), melanization must be subject to NH_4^+-repression in this species. The *Rhizobium* melanization is not necessary for symbiotic nitrogen fixation (Lamb et al. 1982), but *Rhizobium* only fixes N_2 microaerobically. Catechol formation and melanization in strain 184 may be an example of where a chemical protection system has been developed for aerobic nitrogen fixation, and is an alternative to the more traditional enzymatic oxygen protection systems found in obligate aerobes.

3.5 BIBLIOGRAPHY

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CHAPTER 4

Cloning and Expression of the *Azotobacter chroococcum* 184 polyphenol oxidase gene in *E. coli*.

4.1 INTRODUCTION

Organisms of the genus *Azotobacter* are free-living nitrogen-fixing obligate aerobes (Becking, 1981). Among species of this genus, *Azotobacter chroococcum* has been found to be the most commonly isolated from the soils worldwide. Sodium-dependent strains have been recently isolated from the soils of Alberta (Page, 1986). These strains share several characteristics with microaerophilic organisms (Chapter 2; Page et al. 1988). Aeroadaptive, microaerophilic *Azotobacter chroococcum* strain 184 was found to produce a cell-associated pigment when grown at high aeration under nitrogen-fixing conditions. This pigment was identified as a catechol melanin (Shivprasad and Page, 1989; Chapter 3). Although the production of catechol and the melanin appeared to be repressed by ammonium, there did not seem to be any correlation between nitrogen fixation and melanization. Interestingly, the glutamine analog methionine sulfoximine, derepressed catechol and melanin formation, just as it derepresses nitrogenase genes, in the presence of ammonium (Gordon and Brill, 1974). Thus, the production of catechol and in consequence, the melanin, appeared to be regulated in a manner similar to that of the *nif* genes.

Melanins are a class of pigments whose colours range from dark brown to black. These pigments are not essential for the growth and development of the producing species, which include a wide range of microorganisms (Aronson and Wernus; 1965, Prabhakaran et al. 1968; Arai and Mikami, 1972; Beynon et al. 1980; Ivins and Holmes, 1980; Mung and Kondrat'eva, 1981; Sadasivan and Neyra, 1987;), fungi (Fling et al. 1963; Piattelli et al. 1963; Pridham and Woodhead, 1977; Gadd, 1980; Cooper & Gadd, 1984), plants (Robb et al. 1965; Vaughan and Butt, 1970) and animals (Pomerantz, 1963; Burnett, 1971) and insects (Nakamura and Sho, 1964), but increase its survival capabilities in certain environments (Bell and Wheeler, 1986). Melanin, in several microorganisms and fungi, is synthesized from tyrosine via dihydroxyphenylalanine (DOPA). In the Basidiomycetes, melanins are derived from γ-glutaminyl-3,4-dihydroxybenzene (GDHB) (Bell and Wheeler, 1986). Catechol melanins are found in *Ustilago maydis* (Piattelli et al. 1963), and in some fungi, the Ascomycetes, 1,8-

dihydroxynaphthalene (DHN) is the immediate precursor for the melanin (Bell et al. 1976). Thus, melanins are highly complex molecules whose chromophores appear to come primarily from a single biochemical pathway and the final polymer is formed by the condensation of a few similar polyphenol or quinone precursors (Bell and Wheeler, 1986).

Melanin formation is catalyzed by the enzyme tyrosinase, an o-diphenol:oxygen oxidoreductase (or a polyphenol oxidase) which is a copper-containing monooxygenase (Duckworth and Coleman, 1970). It catalyzes the orthohydoxylation of monophenols and aromatic amines to o-diphenols and the o-diphenols to o-quinones. All the subsequent oxidative steps are thought to be nonenzymatic (della-Cioppa et al. 1990). The enzyme has been purified to homogeneity from various sources, viz., *Streptomyces glaucescens* (Lerch and Ettlinger, 1972), *Vibrio tyrosinaticus* (Pomerantz & Murthy, 1974), *Neurospora crassa* (Fling et al. 1963) and mushroom (Bouchilloux et al. 1963), mouse (Burnett, 1971), and *Aspergillus nidulans* (Bull and Carter, 1973).

Melanins can act as "sponges" for free radicals which result from environmental stress. They also increase the resistance of the producing organism to UV irradiation, dessication, and temperature extremes (Bell & Wheeler, 1986). Due to its unique structure, DOPA melanin has been found to act as cation exchange material, as an amorphous semiconductor (McGinness, 1972) and as an amorphous semiconductor threshold switch (McGinness et al. 1974). The binding of drugs such as gentamicin, chloroquine, chlorpromazine and panamycin to melanins in the inner ear and eye of animals has been found to enhance drug efficiency by being selectively toxic to the melanized cells (Ings, 1984; Bell and Wheeler, 1986). Thus there is a growing interest in the production of melanin for use in the drug and cosmetic industry.

In order to study the regulation of melanin production and its role in *Azotobacter chroococcum* strain 184, it is necessary to isolate the polyphenol oxidase gene. As a first step, we have constructed a genomic library of this strain using the broad host range cosmid cloning vector, pLAFR3 (Staskawicz et al. 1987). In this study we report the cloning and expression of the *Azotobacter chroococcum* strain 184 polyphenol oxidase gene in *E. coli*.

4.2 MATERIALS AND METHODS

4.2.1 Bacterial strains and plasmids

Azotobacter chroococcum strain 184 was isolated from Alberta soils (Page, 1986). *E. coli* strains and *Azotobacter* strains used in the study are summarized in Table 4.1a. A map of the cosmid, pLAFR3 (Staskawicz et al. 1987), which was used in the construction of the genomic library is shown in Fig. 4.1. pUC118/119 was used for subcloning (Sambrook et al. 1989). The λDNA packaging kit from Boehringer Mannheim was used for *in vitro* packaging of pLAFR3. A list of plasmids used in the study is summarized in Table 4.1b.

4.2.2 Growth media and growth conditions

The *Azotobacter* strain 184 was routinely subcultured on slants of Burk's nitrogen-free medium containing 0.85 mM NaCl. Liquid medium, pH 7.2, contained 4.6 mM KH_2PO_4, 1.46 mM KH_2PO_4, 0.8 mM $MgSO_4 \cdot 7H_2O$, 1 μM $Na_2MoO_4 \cdot 2H_2O$, 13.2 mM $CH_3COONa \cdot 3H_2O$, and 0.453 mM $CaCO_3$. The nitrogen source was 14 mM ammonium acetate, 2% (w/v) sucrose was used as the carbon source and 14 μM ferric citrate was used as the iron source. The *Azotobacter vinelandii* strains were routinely cultivated on Burk's nitrogen-free medium supplemented with ammonium acetate and sucrose at the same levels mentioned above. The *E. coli* strains were grown in Luria-Bertani (LB) medium (Ohman, 1988) (per litre:10 g tryptone, 5 g NaCl, 5 g yeast extract, pH 7.2). For growth of the nitrogen regulated gene mutants, LB was supplemented with 0.2% glutamine (Prival & Magasanik, 1971). For infection with cosmid, the organism was grown in TB medium (per litre:10 g tryptone, 5 g NaCl , pH 7.2). Lambda broth (per litre: 10 g tryptone, 2.5 g NaCl, 10 g agar) and top agar (per litre: 10 g tryptone, 2.5 g NaCl, 7 g agar) were used in the Tn5 mutagenesis experiments. For the identification of clones producing melanin, they were grown on LB solid medium supplemented with L-tyrosine (1 g/L) and $CuSO_4 \cdot 5H_2O$ (5 g/L). When used, antibiotics were added to media at the following concentrations (μg/mL): ampicillin, 80; kanamycin 40; tetracycline, 12.5. L-tyrosine and the antibiotics were purchased from Sigma Chemical Co., St. Louis, Mo.

The *Azotobacter* strain was grown at 30°C and the *E. coli* strains were grown at 37°C, in gyratory water bath shakers at 250 rpm and 225 rpm, respectively. In order to study the conditions for melanin production in the *E. coli* clones, they were grown in liquid medium under varying aeration rates, using 5-30 mL volumes in 50 mL Erlenmeyer flasks. The effect of temperature was determined by growing the clone at

Table 4.1a Bacterial strains

Organism	Relevant genotype/properties	Source	Reference
A.chroococcum 184	sodium dependent strain	W.J. Page	Page, 1986
A. vinelandii			
UW	wild type	W. Brill	ATCC12837
UW1	*nif*-1	W. Brill	Fisher and Brill, 1969
E. coli			
VCS257	subclone of DP50 *sup*F which is *sup*E44 *sup*F58 *hsd*S3($r_B^-M_B^-$) *dap*D8 *lac*Y1 *gln*V44 Δ(*galuvr*B)47 *tyr*T58 *gyr*A29 *ton*A52 Δ(*thy*A57)	S.E. Jensen	Gigapack λ DNA packaging kit
MV1193	Δ (*lac-pro*AB) *rps*L *thi end*A *spc*B15 *hsd*R4 Δ (*srl-rec*A) 306::Tn10 (tet^r) $lacI^q$	S.E. Jensen	Zoller and Smith, 1987
HB101	*sup*E44 *hsd*S20 ($r_B^-m_B^-$) *rec*A13 *ara*14 *pro*A2 *lac*Y1 *gal*K2 *rps*L20 *xyl*-5 *mtl*-1	S.E. Jensen	Boyer and Roulland-Dussoix, 1969
ED2601	a derivative of JC3272 lac^- his^- trp^- lys^- sm^r $T6^r$ fla^-	L. Frost	Achtman et al. 1971
LE392	*sup*E44 *sup*F58 *hsd*R514 *gal*K2 *gal*T22 *met*B1 *trp*R55 *lac*Y1	L. Frost	Borck et al. 1976
ET8000	*rbs lac*Z::IS1 *gyr*A $hutC_K^c$	D. MacNeil	MacNeil, 1981
ET8052	ET8000:*gln*F100	D. MacNeil	Pahel and Tyler, 1979
ET8894	ET8000:(*rha-gln*G)1703::Mu *cts*62	D. MacNeil	MacNeil, 1981

Table 4.1b Phages and plasmids

Plasmid/Phage	Relevant genotype/properties	Source	Reference
Plasmids			
pLAFR3	tet^r. Derived from IncP-1 plasmid RK2	S.E. Jensen	Staskawicz et al. 1987
pMASS1	pLAFR3 containing 22 kb insert with *ppo* gene of *A. chroococcum* 184	This study	
pMASS2	pLAFR3 containing a 7.13 kb *Bam*HI-*Hind*III insert with *ppo* gene of *A. chroococcum* strain 184	This study	
pMASS3	pUC119 containing 1.87 kb *Bam*H1-*Sst*1 fragment with *ppo* gene of *A. chroococcum* 184	This study	
pUC118/119	amp^r	S.E. Jensen	Vierra and Messing, 1987
pCK1	kan^r, contains *nif*A of *Klebsiella pneumoniae* cloned in pKT230, a hybrid of RSF1010 (Q incompatibility group) and pACYC177	C. Kennedy	Kennedy and Robson, 1983
Phages			
λ::Tn5*tac*1	λ b221 cI857 Oam29 Pam80 with Tn5*tac*1	L. Frost	Chow and Berg, 1988

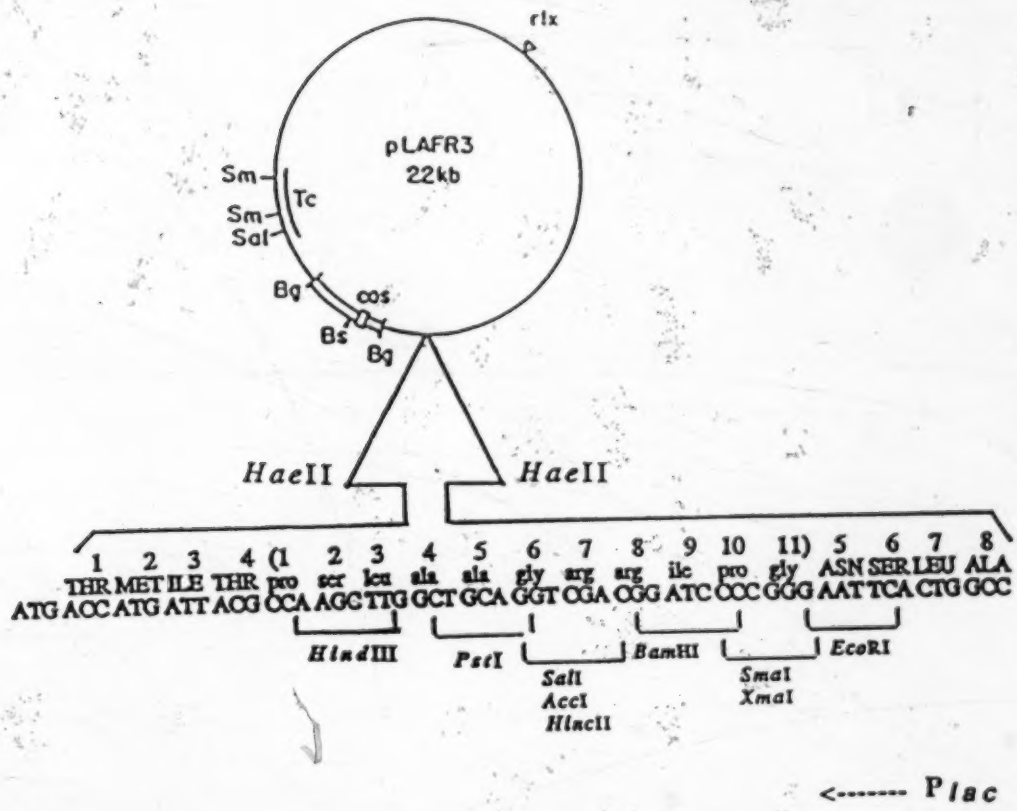

Fig. 4.1. Circular restriction map of pLAFR3 showing the 454 bp *Hae*II fragment from pUC8. The amino acid sequence for the N-terminus of β-galactosidase is presented as a reference. The amino acids shown in lower case letters are those that result from the DNA sequence of the multiple cloning site inserted into the β-galactosidae gene. Adapted from Staskawicz et al. (1987), and the Bethesda Research Laboratory catalogue and reference guide.

30°C and 37°C, and the effect of ammonium was studied by adding 14 mM ammonium acetate to LB-tyrosine medium.

4.2.3 Plasmid isolation and analysis

Plasmids were purified from the *E. coli* strains by using a modified Birnboin and Doly (1979) small scale alkaline lysis technique as described by Sambrook et al. (1989). The organism was grown overnight in liquid medium. 1.5 mL of culture was transferred to a 1.5 mL Eppendorf tube and spun for 5 minutes in a microfuge. The supernatant was discarded and another 1.5 mL of culture was added and spun. The pellet was resuspended in 200 µL of Solution #1 (50 mM glucose, 10 mM EDTA and 25 mM Tris-HCl, pH 8.0). The suspension was mixed well by vortexing and 400 µL of Solution #2 (0.2 M NaOH, 1% (w/v) SDS) was added and the contents mixed gently by inverting the tube. After 5 minutes on ice, 300 µL of solution #3 (3 M sodium acetate solution, pH 4.8, adjusted with glacial acetic acid) was added and the contents mixed by inverting the tube. After 5 minutes incubation on ice, the tube was spun for 10 minutes at 4°C. The supernatant was transferred to a clean Eppendorf tube and 0.75 volumes of phenol added, the contents mixed, and the tube spun for 2 minutes at room temperature. Phenol:chloroform (1:1) extractions were carried out in a similar way, until the interface was clear. At each stage the supernatant was removed and transferred to a clean tube. Finally the DNA was extracted with chloroform, and then ethanol precipitated by adding 1/10 volume 3 M sodium acetate and two and a half volumes of 95% ethanol. The tube was placed at -20°C for at least 1 hour after which the DNA was collected by centrifugation. The DNA was then washed with 70% ethanol to remove salt and then dried, and redissolved in TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA, pH 8.0) (Sambrook et al. 1989). DNAase-free pancreatic RNAase (0.1 mg/mL final concentration) digestion was carried out at 37°C for 1 hour and the DNA stored at -20°C. To obtain DNAase-free RNAase, the procedure described by Sambrook et al. (1989) was used, where, pancreatic RNAase (RNAase A) was dissolved in a solution containing 10 mM Tris-HCl, pH 7.5 and 15 mM NaCl at a concentration of 10 mg/mL. The solution was heated to 100°C for 15 minutes and then allowed to cool slowly to room temperature and dispensed into aliquots which were stored at -20°C. For larger scale plasmid preparations, the same procedure was followed, except that the number of tubes was increased.

4.2.4 Restriction digestion and ligation of DNA

The procedure followed was essentially that of Sambrook et al. (1989). Vector DNA and foreign DNA were cut with the desired compatible restriction enzymes at 37°C. After phenol:chloroform extractions and ethanol precipitation, the DNA was redissolved

in TE buffer. Vector DNA was then added to foreign DNA in a microfuge tube in the desired ratios. The combined DNA was ethanol precipitated, dried and resuspended in a total volume of 7 µL distilled water. The mixture was placed in a 45°C water bath for 5 minutes to melt any cohesive terminii that had reannealed and the mixture was then chilled to 0°C. 2 µL of 5x T4 DNA ligase buffer (Sambrook et al. 1989), 1 µL of 1:10 diluted bacteriophage T4 DNA ligase, and 1 µL 5 mM ATP were added and the reaction allowed to proceed at 12°C for 7 hours. The ligated mixture was ethanol precipitated before being used in the transformation. The 5x DNA ligase buffer contained 100 mM Tris-HCl (pH 7.6), 25 mM $MgCl_2$, 25 mM dithiothreitol and 250 µg/mL bovine serum albumin (fraction V; Sigma).

4.2.5 Electrophoresis of DNA

Electrophoresis of DNA in agarose gels was carried out as described by Sambrook et al. (1989) using TAE buffer (per litre: 4.84 g Tris base, 1.142 mL glacial acetic acid, 2 mL 0.5 M EDTA pH 8.0, water to one litre). The percentage of agarose used was 0.75%, unless otherwise stated. After electrophoresis, the gels were stained with ethidium bromide (1 µg/mL) and destained with distilled water. Photographs were taken using UV illumination and a red-orange filter.

4.2.6 Transformation of *E. coli*

Competent cells of *E. coli* strains VCS257 and MV1193 were prepared according to the procedure of Ohman (1988). *E. coli* was grown overnight in LB liquid medium at 37°C. An aliquot was inoculated into fresh medium (50 mL/ 250 mL flask) for a starting OD_{620} of 0.05. The culture was grown till an OD_{620} of 0.5 was reached. The cells were chilled on ice for 15 minutes and then centrifuged at 1085 x g for 15 minutes at 4°C. The cells were then resuspended in 20 mL cold 100 mM $CaCl_2$ and incubated on ice for 20 minutes. The cells were pelleted by centrifugation at 1085 x g for 10 minutes at 4°C and then resuspended in 0.5 mL cold 100 mM $CaCl_2$. After incubation on ice for 3 hours, the cells were transformed with the plasmid. 100 µL of competent cells were added to chilled 13x100 mm test tubes. The DNA (<10 µL) was added and the tubes allowed to sit on ice for 60 minutes. The tubes were then incubated at 43°C for 2 minutes, after which they were placed on ice for another 2 minutes. The transformed cells were then plated out on LB plates with antibiotics using 3 mL 0.6% agarose overlays (when kanamycin was used). After overnight incubation at 37°C the antibiotic-resistant colonies were picked. Small-scale plasmid preparations, as described above, were carried out on a few colonies to confirm the presence of the plasmid.

4.2.7 Construction of DNA library from *Azotobacter chroococcum* strain 184

4.2.7.1 Isolation of genomic DNA

The procedure of Robson et al. (1984) was used. 350 ml of 19 hour old cells grown in Burk's medium were centrifuged at 10,400 x g for 10 minutes The pellet of cells was washed in PEM (5 mM K_2HPO_4, 0.1 mM EDTA, 0.5 mM $MgCl_2$, pH 8.0) and resuspended in 12 mL of TES (10 mM Tris, 50 mM EDTA, 50 mM NaCl, pH 8.0) and SDS added to 1.5% (w/v). The suspension was incubated for 10 minutes after which Protease K (Sigma Type XI) (to 500 µg/mL) was added and the solution placed at 37°C for 3 hours. The DNA was precipitated with 2 volumes of ACE (sodium acetate, 0.2 M, in 90% v/v ethanol) and collected with a glass rod, drained and redissolved in TE buffer The solution was extracted three times with phenol (at 23°C) and once with chloroform. The DNA was reprecipitated with 2 volumes of ACE and collected on a glass rod and then was dissolved in TE buffer. RNAase digestion was carried out by adding DNAase-free pancreatic RNAase (20 µg/mL) and incubating for 45 minutes at 37°C. To estimate the concentration of DNA the absorbance was determined at 260 nm. The A_{260} of a 50 µg/mL solution of pure double stranded DNA is 1 (Sambrook et al. 1989).

4.2.7.2 Partial restriction endonuclease digestion of chromosomal DNA using *Sau* 3A

In order to determine the conditions for partial digestion of *A. chroococcum* chromosomal DNA, 2 µg DNA was digested in 15 µL total volume with different amounts of *Sau* 3A (from 0.0005 units/µg - 0.005 units/µg DNA and varying incubation times at 37°C. The samples were analyzed by agarose gel electrophoresis (with 0.5% agarose) using bacteriophage lambda DNA fragments, which had been cut to known sizes, as molecular weight markers. The digestion conditions which gave the highest amount of DNA in the 20-25 kb range was determined after staining the gel with ethidium bromide (1 µg/mL) and observation of the fluorescence intensity under ultraviolet light. Thereafter, a scale-up digestion using 75 µg of DNA was carried out using the same enzyme concentration (0.003 units/µg DNA), time (10 minutes), and DNA concentration as was used in the small scale digestion. After ethanol precipitation, the DNA was dried under vacuum and then redissolved in TE buffer. An aliquot was analyzed on a 0.5% agarose gel to confirm the presence of 20-25 kb fragments in the large scale digestion.

4.2.7.3 Isolation of 20-25 kb fragments by sucrose gradient centrifugation

a) Preparation of sucrose gradient. As described in the Microbiology 444 laboratory manual of the University of Alberta, Edmonton, Canada, the sucrose gradient was prepared using 30% sucrose in 1 M NaCl, 20 mM Tris-HCl (pH 7.8) and 10% sucrose in 1 M NaCl, 20 mM Tris-HCl (pH 7.8). A conical chambered gradient making device shown in Fig. 4.2, was used. The following stepwise procedure describes the preparation of the gradient.

i) Pipette 6.5 mL of the 30 % sucrose solution into chamber M of the gradient maker with valve and clamp closed.

ii) Slowly open the valve to allow the concentrated solution to fill the connecting line between the two chambers. There must be no air bubbles in the line. A rapid flow of solution from chamber M to chamber R will flush the air bubbles out. Any concentrated solution in the R chamber may be returned to the M chamber by tilting the gradient maker with the valve open so that the solution flows slowly into the M chamber but leaves the line filled.

iii) Add 6.9 mL of the 10 % sucrose solution into the R chamber.

iv) Position the mixer such that it centrally located with its lower end about 3 mm from the bottom of the M chamber. Begin mixing, avoiding violent agitation.

v) Open the valve fully. Connect the outlet tube to an SW-40Ti centrifuge tube, and open the clamp slowly. Make sure that the less concentrated solution is being mixed thoroughly with the concentrated solution. A suitable flow rate will result in the gradient forming in not less than 10 minutes and not more than 15 minutes.

b) Loading of DNA. The digested DNA, in 0.25 mL of 1M NaCl, 20 mM Tris-HCl (pH 7.8), was carefully loaded on the gradient and the tubes were balanced before placing in the SW-40Ti rotor. The tubes were centrifuged at 35,000 rpm for 20 hours at 4°C.

c) Collection of fractions. After centrifugation for the required time, the tube was removed from the centrifuge, punctured and 30-drop fractions collected into 1.5 mL Eppendorf centrifuge tubes. 50 µL from each alternate fraction was ethanol precipitated for 1 hour at -20°C, centrifuged, dried, redissolved in TE buffer and analyzed by agarose gel electrophoresis on a 0.5% agarose gel. The gradient fractions containing DNA fragments in the size range of 20-25 kb were then pooled, ethanol precipitated, centrifuged, dried and resuspended in TE buffer. The A_{260} of the DNA was then determined.

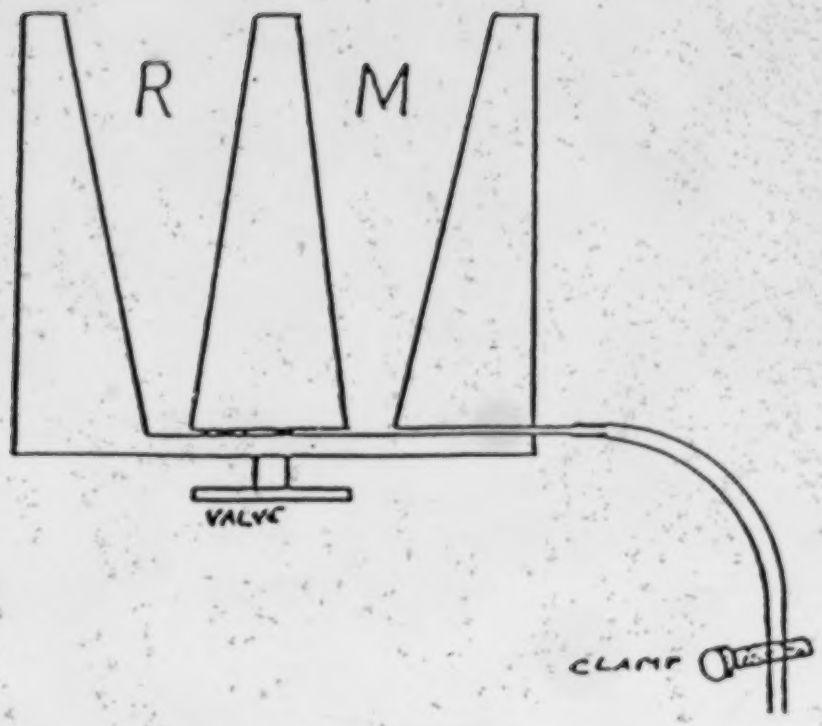

Fig. 4.2 Conical chambered gradient making device used for the preparation of sucrose gradients. Chamber M contains the 30% sucrose solution and chamber R contains the 10% solution.

4.2.7.4 Cutting of pLAFR3 and alkaline phosphatase treatment

pLAFR3 was totally digested with *BamH*I (BRL) to linearize the cosmid vector. An aliquot was analyzed on a 0.5% agarose gel to ensure that the cosmid was cut. For phosphatase treatment to eliminate religation of the cosmid during the ligation step, a portion of cut cosmid was brought up to 100 µL with 50 mM Tris pH 7.2. 1 µL of 1:100 diluted calf intestinal alkaline phosphatase (Boehringer Mannehcim) was added and the mixture incubated at 37°C for 30 minutes after which another microlitre of diluted alkaline phosphatase was added and the reaction allowed to proceed for another 30 minutes. The reaction was then stopped by heat inactivating the alkaline phosphatase at 70°C for 15 minutes. The sample was then extracted once with 50 µL phenol, once with 50 µL chloroform and then ethanol precipitated by adding 1/10 volume of 3 M sodium acetate and 2.5 volumes of 95% ethanol and incubating at -20°C for 1hour. The DNA was then collected by centrifuging at 4°C for 30 minutes, dried and then redissolved in TE buffer. The A_{260} was determined. A sample was run on a gel along with the pooled 20-25 kb chromosomal DNA to estimate the relative amounts of DNA. 0.5 µg of pooled genomic 20-25 kb fragments was ligated to 0.5 µg of cut and phosphatased pLAFR3.

4.2.7.5 *In vitro* packaging of recombinant cosmids

The procedure described in the data sheet of the DNA packaging kit provided by Boehringer Manneheim was followed using 3 µL of ligation mixture. A pipette tip with ultrasonic extract (SE) was placed in an Eppendorf vial with the freeze thaw lysate (FTL) and both were allowed to thaw slowly on ice. During the thawing, the 3 µL of ligated DNA was added, and after completing thawing, the mixture was carefully homogenized. The mixture was incubated at room temperature (22°C) for 1 hour after which the resulting lysate was diluted to 500 µL with a solution containing 10 mM Tris-HCl (pH 7.4), 10 mM $MgSO_4$, 0.01% gelatin (w/v). A drop of chloroform was added and the mixture stored at 4°C.

4.2.7.6 Infection of *E. coli* strain VCS257 with packaged cosmid

E. coli strain VCS257 was grown overnight in TB liquid medium supplemented with 10 mM $MgSO_4$, and 0.2% maltose. The next day, fresh medium with the same supplements was inoculated with the overnight culture for a starting OD_{620} of 0.05. The culture (20 mL/50 mL flask) was grown with shaking at 300 rpm until it reached an OD_{620} of 0.5 (which took approximately 2 hours). The cells were harvested by centrifuging at 6500 x g for 2 minutes. They were washed with 0.4 volumes of 10 mM $MgSO_4$ and then resuspended in SM buffer: per litre, 5.8 g NaCl, 2 g

$MgSO_4 \cdot 7H_2O$, 50 mL 1 M Tris-HCl pH 7.5, 5 mL 2% gelatin (Sambrook et al. 1989). The buffer was sterilized by autoclaving. To 200 µL cells (OD_{620}= 0.5) in a 13x100 mm test tube was added 5 µL packaged cosmid, and the mixture placed at 37°C for 30 minutes for adsorption of phage to the cells. 200 µL of LB medium was added and the tube was incubated on a tube roller at 37°C for 90 minutes to allow for expression. Aliquots were then plated out on LB-tetracycline (12.5 µg/mL) plates using 3 mL 0.6% agarose overlays. After 24 hours the colonies were counted, and using a grid pattern the colonies were spotted on LB-tetracycline plates.

4.2.7.7 Calculations

The genome size of *Azotobacter chroococcum* is approximately 1.94×10^6 kb (Robson et al. 1984). The calculations of Seed et al. (1982) were used to determine the number of individual clones necessary to obtain a gene bank with a 99.99% probability of being complete.

Where, N= number of clones needed; P= probability than any given gene will be present; F= fractional equivalent of the genome in each clone (~20 kb).

$$N = \frac{\log(1-P)}{\log(1-F)} = \frac{\log(1-0.9999)}{\log(1-20/1940)} = \text{approximately 882 colonies}$$

Therefore, based on these calculations a larger scale infection was carried out and 2126 colonies were obtained. The colonies were spotted (using sterile toothpicks and grid patterns) in duplicate onto LB tetracycline plates which were incubated overnight at 37°C.

4.2.7.8 Transfer of colonies to nitrocellulose filters

The procedure described by Sambrook et al. (1989) was essentially followed, with minor modifications. Following overnight growth of *E. coli* containing *Azotobacter* genomic DNA, the colonies were transferred from the master plates to nitrocellulose membrane discs. This was done by placing the membrane on agar plates with the colonies till the membrane became evenly wet. The filter was then lifted with a pair of forceps and placed, colonies on the upper side, on a 3MM Whatman filter paper which was previously saturated with denaturing solution (1.5 M NaCl, 0.5 M NaOH). All the steps in this procedure were carried using pyrex baking dishes to hold the Whatman filters. After 5 minutes, the filter was transferred to a 3MM Whatman filter paper saturated with neutralizing solution (1.5 M NaCl, 0.5 M Tris-HCl, pH 8.0). After 5 minutes, the filter was placed on a third 3MM filter paper saturated with neutralizing solution. The filter was left for 5 minutes. The nitrocellulose membrane was allowed to air dry and then sandwiched between two sheets of dry 3MM Whatman filter paper and baked in a vacuum oven at 80°C for 2 hours. The sandwiched filters were then stored in sealed bags until further use. In addition to using nitrocellulose membranes to preserve

the genomic library, the individual colonies were inoculated into LB tetracycline liquid medium containing 15% glycerol in microtitre plates. Each well was inoculated using a toothpick and the organisms allowed to grow for 2 days after which the microtitre plates were stored at -70°C.

4.2.7.9 Infection of *E. coli* strain VCS257(pCK1) with packaged cosmid

Strain VCS257 was transformed with pCK1 by the procedure described above (in: Transformation of *E. coli*). The same procedure as described above for infection of VCS257 with the packaged cosmid was followed (4.2.7.6 above). The clones were plated out on LB plates containing tetracycline (12.5 µg/mL) and kanamycin (40 µg/mL). 1600 colonies were screened for melanin producing clones.

4.2.8 Identification of melanin-producing clones and subclones

E. coli VCS257 colonies infected with recombinant pLAFR3 were spotted on LB-tetracycline plates supplemented with 1 mg/mL L-tyrosine and 5 µg/mL $CuSO_4 \cdot 5H_2O$. LB-kanamycin-tetracycline plates supplemented with L-tyrosine (0.5 mg/mL) and $CuSO_4 \cdot 5H_2O$ (5 µg/mL) were used to identify *E. coli* VCS257 (pCK1) colonies containing recombinant pLAFR3. The colonies were grown up until brown colour was observed in the medium surrounding the colonies. *E. coli* strain VCS257 containing recombinant pUC plasmids were grown in LB liquid medium containing tyrosine, copper and isopropyl-β-D-thiogalactopyranoside (IPTG; 1mM) as inducer.

4.2.9 Isolation and estimation of melanin

For the quantitative estimation of melanin, the cells were grown in LB medium (200 mL/500mL flask) supplemented with tyrosine and copper sulphate. The melanin was isolated as previously described (Shivprasad & Page, 1989; Chapter 3). The only difference in the isolation procedure was that since the melanin was isolated from culture supernatants, the first step in the isolation involved lyophilization. After extraction, the residual material was resuspended in 0.85 N KOH and heated to 100°C for 5 minutes. After centrifugation to remove debris, the supernatant was used for the estimation of melanin. For the estimation of melanin, the procedure of Rosenthal et al. (1973) was used. Here, the melanin in a volume of 1 mL was heated to 100°C for 30 minutes in the presence of 50 µL of 30% H_2O_2. The samples were then cooled to room temperature and the fluorescence emitted at 500 nm was estimated by excitation at 410 nm using a Hitachi F-2000 fluorescence spectrophotometer. DOPA-melanin from Sigma Chemical Co, was used as the standard. A calibration curve was prepared by using 1-50 µg/mL Sigma melanin dissolved in 0.85 N KOH. Protein was estimated by the procedure of Bradford (1976).

4.2.10 Preparation of cell-free extracts and estimation of tyrosinase activity

The cells were grown in LB medium supplemented with tyrosine and copper for 24 hours. The culture was spun at 10,400 x g for 10 minutes to collect the cells. After resuspension in 1 mL 0.5 M phosphate buffer (pH 6.8), the cells were sonicated with a Braun Sonic 2000. The cells were disrupted by four 20-s bursts of full power at 1-minute intervals. The suspension was treated with RNAase (10 µg/mL) and DNAase (10 µg/mL) for 30 minutes at room temperature. Cell debris was removed by centrifugation at 1935 x g for 20 minutes and the supernatant saved.

Tyrosinase activity was measured using the dopachrome assay of Lerch & Ettlinger (1972). DL-DOPA was used as the substrate (10 µmole) in sodium phosphate buffer (0.1 M, pH 6.8). Enzyme and additional buffer was added to a total volume of 1.0 mL and the formation of dopachrome monitored continuously for 15 minutes by measuring the increase in absorbance at 475 nm using a Hitachi Model U-2000 double-beam spectrophotometer. The molar extinction coefficient for the reaction at 475 nm is 3600 M^{-1} cm^{-1}.

4.2.11 Restriction mapping

The location of sites cut by various restriction enzymes was determined by cutting the recombinant cosmid with an array of restriction endonucleases as described by Ohman (1988). The cosmid was cut with single restriction enzymes, then to orient the fragments, double digests were performed with two restriction enzymes. Using a graphical method, the sizes of the DNA restriction fragments were determined. The fragments generated when λ DNA was cut with *Hind*III and *Bst*EII were used as size standards. By plotting the logarithmic value of molecular size vs. mobility of the standards, the size of the fragments was determined.

4.2.12 Subcloning

For subcloning, pUC 118 and pUC 119 were used. For this procedure, double digests of the vector and cosmid containing the insert were carried out, and after phenol:chloroform and chloroform extractions to remove the restriction enzymes, the DNA was ethanol precipitated. Ligations were carried out using equal amounts of vector DNA and insert DNA, and *E. coli* strain MV1193 (the host for pUC vectors) was transformed with the ligated DNA. Screening for pUC with inserts was carried out as described by Sambrook et al. (1989) where 10 µL of IPTG (30 mg/mL) and 20 µL of X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) (20 mg/mL) were spread on the surface of LB-amp plates prior to plating out the transformed cells. After incubation of the plates for 12-16 hours at 37°C, they were placed at 4°C for a few hours to develop the

blue colour fully. Plasmid preparations were carried out on the subclones and the size of the inserts estimated by gel electrophoresis. Constructions were also made with the polyphenol oxidase gene in the opposite orientation so that the *lac* promoter of the vector was downstream of the gene. The plasmids with the subcloned inserts were used to transform *E. coli* strain VCS 257.

4.2.13 Curing pCK1 from the melanin producing *E. coli* clone

The *E. coli* strain was grown for repeated generations in LB liquid medium lacking kanamycin. This was carried out in 10 mL flasks containing 3 mL medium. At each transfer, 20 µL of the culture was inoculated into fresh medium. At each transfer dilutions were prepared and aliquots were plated out on LB-kan-tet and LB-tet plates to determine the relative numbers of kan-resistant colonies remaining. After 17 such transfers, three kan-sensitive colonies were spotted on LB-tetracycline plates supplemented with L-tyrosine and copper sulphate to determine the production of melanin. Of these one was found to lack pCK1 and also produced melanin. Plasmid DNA from this clone was isolated and used to transform *E. coli* strain VCS257.

4.2.14 Plasmid mobilization

Transfer of pMASS1 (pLAFR3 containing the 22 kb chromosomal DNA insert of strain 184) into *Azotobacter vinelandii* UW and UW1 was accomplished by triparental mating using pRK2013 as the mobilizing transfer factor (Figurski and Helinski, 1979). The procedure followed was essentially that of Ruvkun and Ausubel (1981) with minor modifications. *E. coli* strains VCS257(pMASS1) and HB101(pRK2013) were grown overnight in LB-tet and LB-kan, respectively. Fresh media were inoculated from the respective overnight cultures, and the organisms incubated (20 mL medium/50 mL flasks) until the OD_{620} was about 0.5. *Azotobacter vinelandii* strains UW and UW1 (Nif^- strain) were inoculated into Burk's medium containing glucose and ammonium acetate (BBGN). After 20 hours of growth, the cells were harvested by centrifuging at 3020 x g for 10 minutes at 4°C. The *E. coli* cultures were harvested at the same time. The cells were washed with PEM and then resuspended in 1 mL Burk's buffer. The OD_{620} of the cultures were determined. Using the assumption that the size of the *Azotobacter* cell is about double that of the *E. coli* cells, the cultures were mixed so as to have approximately the same number of each of the three types of organisms. The cells were pelleted by centrifugation, and then resuspended in 0.1 mL Burk's medium. The whole volume was spread on BBGN plates and the plates incubated at 30°C. At regular intervals, up to 45 hours, a loopful of culture was scraped off and was plated out on BBGN-tet plates. Of the colonies which developed, four were plated out on BBGN-tet plates supplemented with L-tyrosine and copper sulphate to screen for melanization.

4.2.15 Transposon Tn5 mutagenesis

Tn5 mutagenesis of the cloned gene was carried out using a derivative of Tn5, Tn5*tac*1 (Chow and Berg, 1988) (Fig. 4.3) *E. coli* strain ED2601 was first transformed with pMASS1. Transposition of Tn5*tac*1 into pMASS1 then was obtained by infecting ED2601pMASS1(Sup$^-$) with λ::Tn5*tac*1 and selecting kanamycin resistant transductants. The procedure of Way et al. (1984) was used for phage infection. In this procedure, after overnight growth of *E. coli* ED2601 (pMASS1), the culture was inoculated into fresh lambda broth-tetracycline medium containing 0.2% maltose and 10 mM magnesium sulphate. The culture was incubated until an OD_{620} of 0.4 was reached (~$3x10^8$ cells /mL). Phage was added to get an moi (multiplicity of infection) of 0.3 per cell (the phage titre was determined in advance using *E. coli* strain LE 392 as the host). After incubation for 30 minutes at room temperature to permit adsorption, the flasks were incubated in a water bath shaker at 225 rpm at 37°C for 23 hours. The cells were then collected by centrifugation at 10,400 x g for 10 minutes and resuspended in Burk's buffer. The cells were plated out on lambda plates (see above: Growth media and growth conditions) containing kanamycin and tetracycline, using 3 mL top agar overlays. The colonies were screened for melanin production on LB-tyrosine-copper plates with and without IPTG (0.5 mM).

4.3 RESULTS

4.3.1 Construction of genomic library and isolation of clone expressing polyphenol oxidase activity in *E. coli*

An *Azotobacter chroococcum* 184 chromosomal library with an insert size of about 22 kb in pLAFR3 was constructed. During the first library contruction using *E. coli* VCS257 as a host, from among 2126 colonies, no melanin-producing clone was identified. Since in *Azotobacter chroococcum* strain 184, melanin formation appeared to be regulated by ammonium levels in the growth medium (Shivprasad & Page, 1989; Chapter 3), it was thought that the expression of melanin could require an activator, in the form of the NifA protein which is involved in the activation of nitrogen-fixing genes under derepressing conditions. Therefore, *E. coli* VCS257 was transformed with pCK1, a wide host range multicopy plasmid carrying the *nif*A gene which is expressed from a constitutive promoter. The infection with packaged recombinant cosmid was repeated and from among 1600 colonies, three had brown pigmentation in the medium surrounding the colonies. Of these three, two lost their ability to produce pigment on

Fig. 4.3 Tn5*tac*1. Near the right end of this 4.6 kb element is the P_{tac} promoter, facing outward. "I" and "O" correspond to inner and outer sequences which are the first 19 base pairs from each end of IS50. The *lacI*^{q} allele directs the synthesis of high levels of repressor and therefore ensures tight regulation of transcription from P_{tac}. Restriction sites and their positions in base pairs from I are as follows: E, *Eco*RI, 25 and 4558; C, *Cla*I, 52; S, *Sma*I, 1384 and 4563; A, *Apa*I, 2110; H, *Hind*III, 3165; N, *Not*I, 3666; X, *Xho*I, 3775; and B, *Bam*HI, 4254 and 4565. Abbreviations: kan, kanamycin resistance; tnp, transposase. Copyright permission for the figure which was reproduced from Chow and Berg (1988) could not be obtained.

subculturing. The melanin-producing clone, *E. coli* VCS257(pMASS1), contained an insert of about 22 kb.

4.3.2 Melanin production by *E. coli* VCS257pCK1(pMASS1)

The melanin-producing clone was grown in a range of aeration conditions which were imposed by shaking different volumes of medium at 225 rpm (data not shown). Pigment production was higher at the higher aeration than at the lower aeration. The effect of temperature was studied by growing the organism at 37°C and 28°C since the *nif*A-dependent activation of *nif* transcription is temperature-sensitive and no induction of transcription occurs at temperatures higher than 35°C (Buchanan-Wollaston et al. 1981). Plates and liquid media incubated at 37°C, however, demonstrated more pigmentation than those incubated at 30°C (data not shown). To determine the effect of ammonium on pigmentation as is seen in the *Azotobacter*, the organism was grown in LB medium containing 14 mM ammonium acetate. Higher levels of ammonium did not repress melanin formation. Therefore, the effect of the NifA protein in regulation of melanin formation was not evident.

4.3.3 Melanin production in nitrogen regulated mutants of *E. coli*

In order to further study the regulation of melanin formation and determine the role of the *ntr* genes in melanin formation in *Azotobacter chroococcum* strain 184, pMASS1 was introduced into *E. coli* strains ET8052 (NtrA$^-$), ET8894 (NtrC$^-$) and ET8000 (wild type). NifA mediated activation of gene transcription requires a functional *ntr*A gene which encodes the σ^{54} subunit of RNA polymerase, thus allowing the enzyme to recognize *nif* promoters (Hunt & Magasanik, 1985; Hirschman et al. 1985). Since in *Klebsiella pneumoniae*, the product of *ntr*C is required to activate transcription of *nif*A, the effect of *ntr*C$^-$ mutations was also investigated. All three strains were transformed with the NifA plasmid pCK1 and with pMASS1 Thus the effect of *ntr* mutations in the presence and absence of *nif*A were studied. However, from these studies it was observed that there was no effect on melanin formation and all three strains produced the same amount of melanin whether *nif*A was present or not. Thus it was further confirmed that melanin formation did not require a functional NifA in *E. coli*.

4.3.4 Curing of *E. coli* VCS257pCK1(pMASS1)

Since the effect of the NifA protein was not apparent in the activation of melanin formation, it was proposed to cure the clone from the *nif*A-containing plasmid, pCK1. After 17 transfers of the clone in medium lacking kanamycin, three colonies which were kanamycin-sensitive were tested for melanin production. Of these, one was found to produce melanin and on analysis of its plasmid profile, lacked pCK1. The other two which had lost the ability to produce melanin had also lost pMASS1. To eliminate the

possibility that pCK1, in the melanin-producing clone, might still be present after integration into the chromosome, pMASS1 was isolated from the organism and used to transform *E. coli* VCS257. *E. coli* VCS257 (pMASS1) produced melanin on LB medium with tyrosine and copper sulphate, thus eliminating the possibility of the requirement of NifA for melanin expression in the clone.

4.3.5 Melanin production in *E. coli* VCS257(pMASS1)

E. coli strain VCS257(pMASS1) was grown in LB liquid medium and in the same medium supplemented with L-tyrosine and copper sulphate. It was evident that tyrosine enhanced pigmentation. The melanin was not cell-associated as in the original *Azotobacter* strain, but was found in culture supernatants (Fig. 4.4) and in the medium surrounding the colonies. The melanin was isolated by the procedure described in Chapter 3. The spectral characteristics of the cloned melanin, which is a DOPA-melanin, was identical to that of Sigma melanin on treatment with H_2O_2 as described in Materials & Methods (Fig. 4.5).

4.3.6 Subcloning, mapping and expression of the polyphenol oxidase (*ppo*) gene

Using the restriction map generated for pMASS1 (Fig. 4.6) subcloning of the DNA fragment was carried out by the standard procedures described in Sambrook et al. (1989). The restriction sites on this cosmid were mapped and further subcloning was carried out using pUC118 and pUC119 as cloning vectors. Restriction mapping of pMASS1 demonstrated that the 22 kb insert had one *BamH*I-*Hind*III fragment of about 15 kb and another of about 7 kb (Fig. 4.6). *Hind*III digestion of pMASS1 resulted in the formation of two fragments, one of about 15 kb in length and the other of about 27 kb (which included about 7 kb of insert DNA). The plasmid was cut and the linearized DNA was used to transform *E. coli* VCS257. All of 14 transformants produced melanin. On analysis of their plasmid content and size by agarose gel electrophoresis, four appeared to have plasmids smaller than the original plasmid. A double digest of one of these was carried out using *BamH*I and *Hind*III and the size of the insert was found to be 7.13 kb in length. This plasmid was renamed, pMASS2 (Fig 4.6). Melanin production was constitutive in this strain. LB medium supplemented with tyrosine (5.5 mM) and copper (20 μM) supported melanization (Table 4.2) which was observed even in 18 hour old cultures. For further subcloning, detailed restriction mapping of the 7.13 kb fragment of pMASS2 was carried out to generate the restriction map in Fig. 4.6. For this purpose, single and double digestions with several restriction enzymes were analysed on agarose gels (Fig. 4.7).

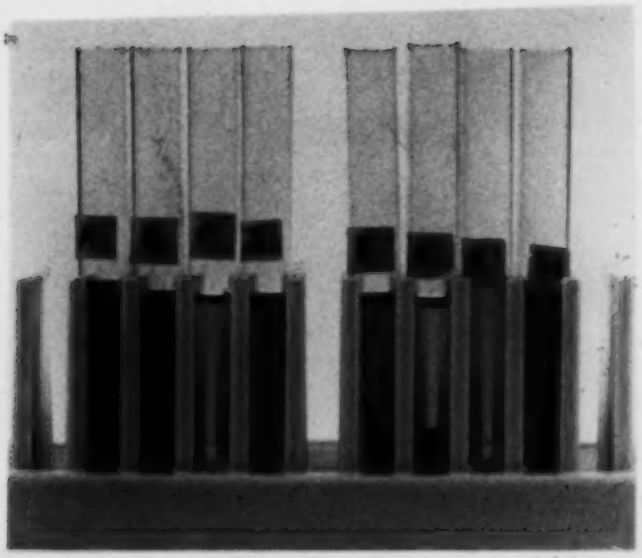

Fig. 4.4. Melanin production in strain 184 under nitrogen-fixing conditions (A), in the presence of copper under nitrogen-fixing conditions (B), *E. coli* VCS257(pLAFR3), the control culture, in LB medium supplemented with tyrosine and copper as described in Materials and Methods (C), and in *E. coli* VCS257(pMASS2) (D) in the same medium described for (C), above. The tubes on the right contain the same sequence of cultures (A to D) described above, except that they have been centrifuged to show the cell-associated melanin in strain 184 and the presence of the melanin in the culture supernatants in the *E. coli* clone containing pMASS2.

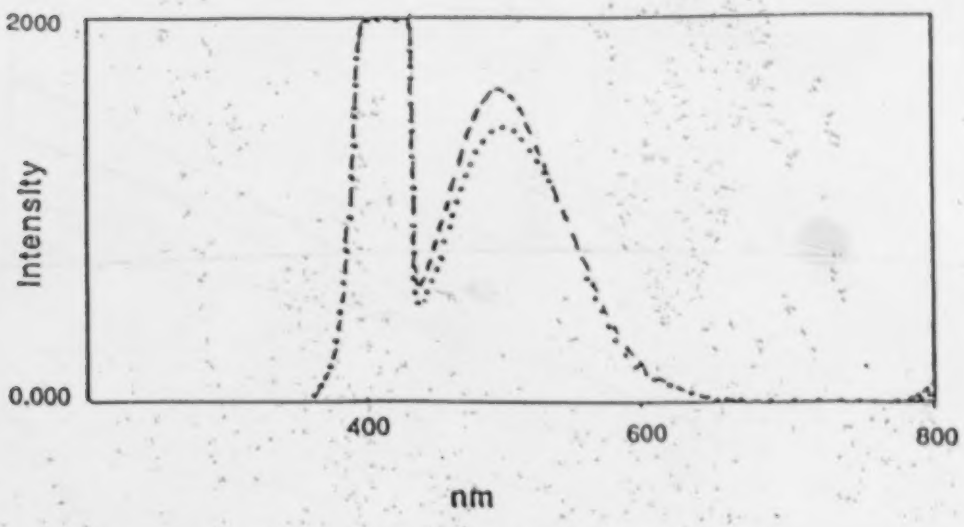

Fig. 4.5. Emission spectrum of Sigma DOPA-melanin (....) and the cloned *A. chroococcum* 184 melanin (----) after heating with H_2O_2 for 30 minutes at 100°C. Excitation wavelength was 410 nm. The melanin was purified from culture supernatants as described in Materials and Methods.

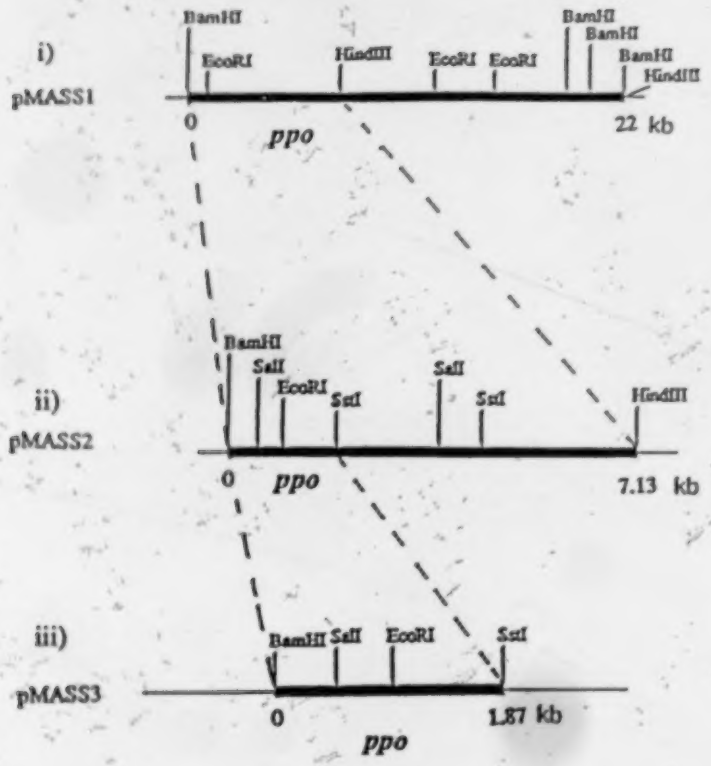

Fig. 4.6. i) Restriction map of pMASS1 which contains a 22 kb insert of chromosomal DNA from *A. chroococcum* strain 184. ii) Plasmid pMASS2 was derived from pMASS1, by cutting pMASS1 with *Hind*III and using the linearized DNA for transformation of *E. coli* as described in Results. The size of the insert is 7.13 kb. iii) Plasmid pMASS3 was derived by subcloning the 1.87 kb *Bam*HI-*Sst*I fragment from pMASS2 into pUC119.

Table 4.2 Melanin production in *E. coli* from the cloned *ppo* gene[a]

Medium[b]	Strain	Protein[c]	Melanin[d]
LB	VCS257(pMASS2)	630	8.2
LB-Tyr-Cu	VCS257(pMASS2)	600	23
LB-Tyr-Cu	VCS257(pLAFR3)	590	1.9

a All values are means of at least two duplicate determinations. Melanin was estimated after 48 hours incubation as described in Materials and Methods.

b LB: Luria-Bertani medium as described in Materials and Methods. Tyrosine (1 mg/mL) and copper sulphate (5 µg/mL) were added as supplements where indicated.

c Expressed as microgram protein milliliter^{-1}.

d Expressed as milligram melanin (milligram of protein)$^{-1}$.

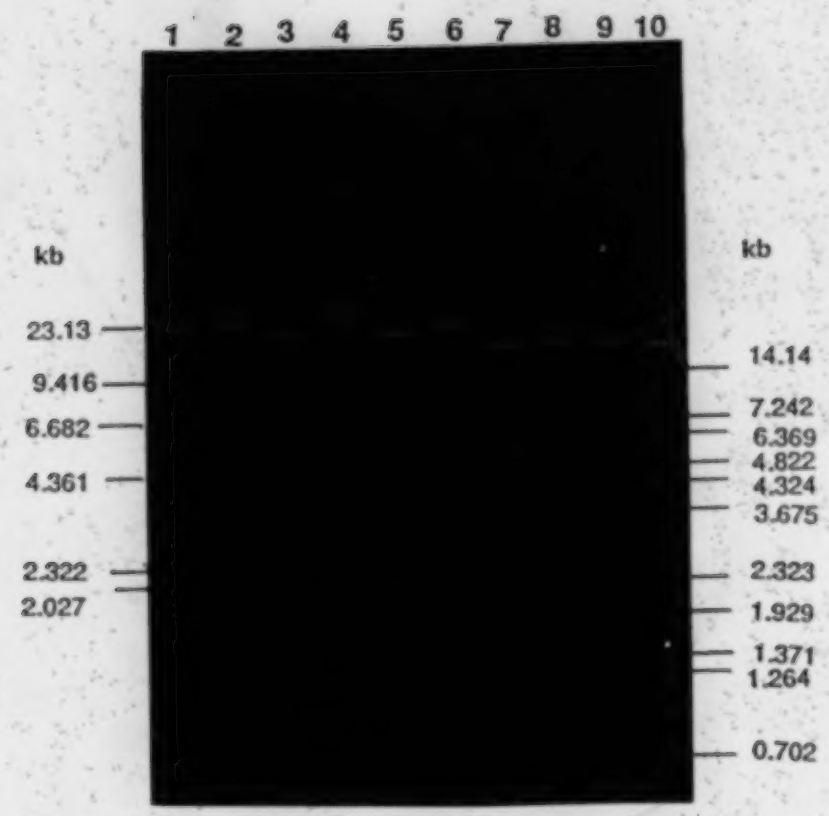

Fig. 4.7. Restriction mapping of pMASS2: analysis by agarose gel electrophoresis. The cosmid was cut with single restriction enzymes and double restriction digests were also carried out to orient the fragments. The enzyme(s) were: *Bam*HI and *Eco*RI (Lane 1), *Eco*RI (Lane 2), *Eco*RI and *Hind*III (Lane 3), *Hind*III (Lane 4), *Hind*III-*Bam*HI (Lane 5), *Bam*HI (Lane 6), *Bam*HI and *Sal*I (Lane 7), *Sal*I (Lane 8), *Hind*III and *Sal*I (Lane 9), and *Sal*I and *Eco*RI (Lane 10). The positions of the size standards are indicated.

In order to determine whether the (*ppo*) gene was under the control of its own promoter or that of the vector, the 7.13 kb *BamH*1- *Hind*III fragment was cloned into the multicopy vectors pUC118 and pUC119. In pUC118, the cloned gene would be in-frame with the *lac* promoter, whereas, in pUC119 the *lac* promoter would be in the opposite orientation (Sambrook et al. 1989). Final subcloning involved the use of double digestions to orient the fragments. Transformants of *E. coli* MV1193 containing plasmids with inserts were identified by the presence of white colonies on plates containing IPTG and X-gal. These colonies were picked up at random and inoculated into liquid media for small-scale plasmid preparations. The colonies were simultaneously spotted onto LB-amp-tyrosine-copper plates to isolate subclones with the *ppo* insert. From these studies it was evident that the *ppo* gene was under the control of the *lac* promoter as it was only expressed in pUC118 and not in pUC119. Therefore, further subcloning in the pUC vectors ensured the correct orientation of the foreign DNA relative to the *lac* promoter. After initial screening for melanin production and isolation of subclones in strain MV1193 producing melanin, it was observed that the ability to produce melanin was lost. Addition of 1 mM IPTG (since MV1193 is a *lacI*^{q} strain) to the medium to derepress the *lac* promoter did not initiate melanin formation. However, when the same plasmids were introduced into strain VCS257 melanin production was observed in medium containing higher amounts of copper sulphate (0.2 mM as against 20 μM) and in the presence of 1 mM IPTG. In the absence of IPTG, melanization was also observed, although it was delayed. Production of melanin by the same strain harbouring pMASS2 however, did not require IPTG or high copper levels even though the gene was being expressed from the *lac* promoter of the vector, here pLAFR3. The smallest fragment on which the *ppo* gene was present and was expressed in *E. coli* was a 1.87 kb *BamH*1-*Sst*1 in pUC119 which was called pMASS3 (Fig. 4.6).

Melanin production by VCS257(pMASS2) was also tested on LB agar plates supplemented with the L-tyrosine analogs N-acetyl-L-tyrosine, and L-tyrosine ethyl ester. Both these analogs supported melanization. The former produced a yellow melanin and the latter a reddish-brown melanin. The control *E. coli* strain, VCS257(pLAFR3) however, did not show any colour changes on these media.

Cell-free extracts of *E. coli* strain VCS257(pMASS2) were prepared for the determination of polyphenol oxidase activity. The control strain, VCS257(pLAFR3) showed an activity of 1.2 nmoles DOPA oxidized per minute, whereas the melanin-producing strain had an activity of 2.10 nmoles DOPA oxidized per minute. In both cases, the amount of protein used in the assay was approximately the same (~4.6 mg protein). The cell extracts which were prepared for the assay were very concentrated

since 200 mL of a 24 hour old culture was resuspended in 1 mL of buffer before sonication. Thus an excessive amount of cellular protein was needed to demonstrate tyrosinase activity, although the cells melanized so actively. Another anomalous behaviour was observed in the analysis of the enzyme by non-denaturing polyacrylamide gel electrophoresis. After staining with DOPA no activity band was observed with the *E. coli* clones (either in the cell extracts or in the culture supernatants) producing melanin. Similar results were obtained on analysis of cell-free extracts of *Streptomyces lividans* (pIJ702), where no activity bands were observed, although melanization was demonstrated. However, the *Azotobacter* strain demonstrated activity bands when analyzed in a similar manner (Chapter 3). It is possible that the cloned gene product was not stable under the conditions used during the electrophoresis.

4.3.7 Melanin production in *A. vinelandii*

Triparental mating experiments were carried out with *A. vinelandii* strains and *E. coli* VCS257(pMASS1) using pRK2013, a conjugative (Tra^+) plasmid. pLAFR3 is not self transmissible, but it can be mobilized at high frequency by using a helper plasmid (Ditta et al. 1980). The expression of the *ppo* gene was studied in *A. vinelandii*, since this species is phylogenitically closer to *A. chroococcum* than *E. coli*. *A. vinelandii* UW1 is a $NifA^-$ strain and therefore it was thought that should the *ppo* gene have its own promoter, and if it was NifA-regulated, this effect should be seen in this strain. Since pLAFR3 is a broad host range cloning vector, it can be transferred to a wide range of organisms by conjugation using the helper plasmid, pRK2013 (Ohman, 1988). Tetracycline-resistant transconjugants were obtained after 15 hours of mating. They were plated out on Burk's medium supplemented with L-tyrosine and copper. Melanization was observed after one week on plates. The colonies were transferred into liquid media of the same composition (15 mL/50 mL flask) and melanization was observed in four days. The melanin was present in the growth medium supernatant and was not cell associated as seen in the original *A. chroococcum* strain. Also, unlike the melanin in *A. chroococcum* which was a catechol melanin (Shivprasad & Page, 1989; Chapter 3), the melanin in *A. vinelandii* was a DOPA-melanin which resulted from the polymerization of the L-tyrosine added to the growth medium. The $NifA^-$ *A. vinelandii strain* UW1 also expressed the *ppo* gene. Since *A. vinelandii* does not have a regulatory mechanism for *lac* operon regulation, it is likely that in the *A. vinelandii* strains, the *ppo* gene of *A. chroococcum* strain 184 was being expressed from its own promoter and not the *lac* promoter.

4.3.8 Effect of copper on melanization

In *A. chroococcum* strain 184, melanization was repressed by ammonium, although, in the presence of exogenous copper added to the growth medium, melanization was observed (Shivprasad & Page, 1989; Chapter 3). Thus copper appeared to reverse the repressive effect of NH_4^+. In all cases examined, the *ppo* gene is a copper-containing metalloenzyme and requires copper for its activity (Duckworth and Coleman, 1970). This requirement for copper was also observed in this study with the multicopy plasmid pUC where multiple copies of the gene, and hence increased levels of gene product, required higher levels of copper (0.2 mM) for activation of the enzyme. In contrast, when the *ppo* gene was cloned in pLAFR3, which was present at only 4 copies per chromosome (Ohman, 1988), the enzyme was activated by 20 μM copper sulphate. In a similar situation with ammonium-grown *A. chroococcum* strain 184, there was no melanization, but there was more cell growth than under nitrogen-fixing conditions in the same period of time. Therefore, it is possible that the amount of available copper was less and hence the percentage of active enzyme in the cell-free extracts was less in the ammonium-grown cells. When extra copper was added (2.5 μM CuCl) to ammonium-grown cells (since no copper was added to nitrogen-fixing cells) there was sufficient copper for the enzyme to be active.

4.3.9 Isolation of Tn5*tac*1 insertion mutations

In order to study the effect of mutations in the *ppo* gene on the growth of *A. chroococcum* strain 184, it was necessary to first mutate the gene and then reintroduce it into the original strain by homologous recombination as described by Ruvkun & Ausubel (1981). To do this Tn5 mutagenesis was carried out. Tn5*tac*1 (Fig. 4.3) was delivered into *E. coli* by infecting with λ::Tn5*tac*1. The phage used was integration-deficient and defective in replication in a non-suppressing host (Chow & Berg, 1988). Thus the kanamycin-resistant transductant resulted through the transposition of Tn5*tac*1 into the chromosome or plasmid of the recipient cell. Fourteen kan-resistant colonies, which resulted from the infection of *E. coli* strain ED2601(pMASS1) with λ::Tn5*tac*1, were screened for melanin production. Tn5*tac*1 contains a strong P_{tac} promoter facing outward, near one end, to promote the transcription of adjacent target DNA, and a *lacI*^{q} gene so that transcription from P_{tac} is tightly regulated (Fig. 4.3). Therefore, insertion of Tn5*tac*1 into a promoter sequence could be identified by growing the mutants in the presence of IPTG, since the expression of the downstream genes would now be under the control of the P_{tac} promoter of the transposon. Nine of the fourteen mutants isolated had the Tn5*tac*1 inserted in the promoter and five were structural gene mutations which completely shut off melanin production. The position of the transposon in the various

mutant genes was not determined. From the subcloning experiments above, it was clear that the expression of the *ppo* gene was under the control of the *lac* promoter of the vector and not its own promoter. Therefore, the promoter mutations had arisen from Tn5*tac*1 insertion into the *lac* promoter of the vector. To confirm the presence of an *Azotobacter* promoter in front of the *ppo* gene, the plasmid carrying Tn5*tac*1 in the vector promoter was introduced into *A. vinelandii* strain UW. Since melanin formation was observed in *A. vinelandii* strain UW carrying the mutated *lac* promoter in front of the *ppo* gene, the presence of the gene's own promoter was confirmed.

4.4 DISCUSSION

Azotobacter chroococcum strain 184 is a sodium-dependent, microaerophilic, nitrogen-fixing organism (Page, 1986; Chapter 2; Page et al. 1988). The characteristics of this organism differ very much from those of other strains of this species (Page, 1987) and recent work combined with 16S rRNA comparisons (E.R.B. Moore, G.E. Fox, and P. Jurtshuk, Jr. Abstract # 251, p.196: Abstracts of the 90th Annual Meeting of the American society for Microbiology, Anaheim, California, 13-17 May, 1990) suggests that this is a new species of *Azotobacter*. Recent studies have demonstrated the production of catechol and melanin when the organism is grown under nitrogen-fixing conditions (Shivprasad & Page, 1989; Chapter 3). The production of catechol and melanin are repressed by ammonium. The level of production of these compounds appeared to be related to the degree of oxygen stress of the organism. It was therefore proposed, due to the properties of melanins to act as free radical scavengers (Mason et al. 1960), that the catechol and the melanin play a role in oxygen protection in this strain which lacks a functional catalase, an essential enzyme for aerobic organisms.

Attempts have been made to mutagenize the *ppo* gene in this strain using various techniques, so that we could study the role of melanin in this organism and the regulatory factors involved. However, due to the failure of these experiments it was proposed to construct a genomic library of the organism in *E. coli*. From this library, the *ppo* gene would be isolated by isolating a melanin producing clone, and the regulatory mechanisms involved in melanin production investigated. For this purpose, a broad-host range cosmid cloning vector, pLAFR3 was used for construction of the library (Staskawicz et al. 1987). This cosmid is 22 kb in size has a tetracycline resistance gene, as a selectable marker, has the *cos* site of phage λ, therefore allowing the construction of gene banks in *E. coli* from large, partially digested fragments of chromosomal DNA (Staskawicz et al.

1987; Ohman, 1988). It has an *ori*T sequence which permits its mobilization by a conjugative plasmid from *E. coli* to other species (Ohman, 1988). Also, it has a low copy number, about 4 copies per chromosome, and therefore, its expression would mimic more closely expression from the chromosome, than if it were replicating at a high copy number (Ohman, 1988).

During the initial screening of the library, no melanin-producing clone was isolated. In *Rhizobium leguminosarum* biovar *phaseoli*, the gene for melanin synthesis has been cloned into *E. coli*, and the production of melanin is activated by *nifA* (Hawkins and Johnston, 1988). Since the *Rhizobium* species is also a nitrogen-fixer, it was speculated that the production of melanin in the *Azotobacter* strain might also be *nifA* regulated. Also, the regulation of catechol and melanin formation by ammonium seemed to fit well with the possibility of *nifA* regulating the *ppo* gene in *Azotobacter*. This appeared to be confirmed by the failure to isolate melanin-producing clones in the first library constructed. Therefore, in the second construction of the genomic library, the *nif*A gene, on pCK1 was delivered into the *E. coli* strain before infection with packaged cosmid. Since melanin- producing clones were isolated, further studies to confirm the role of NifA in regulating melanin formation were carried out. Firstly, unlike the temperature-sensitive *nifA*-mediated regulation of the tyrosinase gene of *Rhizobium* cloned in *E. coli* (Hawkins and Johnston, 1988), the production of melanin by *E. coli* containing cloned *ppo* gene from *Azotobacter* was not temperature-sensitive. In *K. pneumoniae*, *nif*A dependent activation of *nif* transcription is temperature-sensitive, no induction occurs at temperatures higher than 35°C (Buchanan-Wollaston et al. 1981) and therefore the *nif*A gene of *K. pneumoniae* on pCK1 (Kennedy and Robson, 1983) also displayed temperature-sensitivity. Since *nif*A-mediated regulation of *nif* genes requires a functional *ntr*A gene (Hunt and Magasanik, 1985), the expression of melanin formation in an *ntr*A$^-$ strain harbouring the *nif*A plasmid would give insight into the requirement for *nif*A in melanin formation. When the plasmid, pMASS1 was introduced into *E. coli* strains with mutations in the nitrogen-regulated genes, the level of expression in all the strains, viz. *ntr*A$^-$, *ntr*C$^-$ was the same whether or not the *nif*A plasmid was present. Thus the role of *nif*A or *ntr* genes in the regulation of melanin formation directed by the cloned *ppo* gene was not apparent. However, subsequent studies demonstrated that the cloned *Azotobacter* gene was under the control of the *lac* promoter of the vector in *E. coli*. Thus the regulatory mechanisms which were functional in the original *Azotobacter* strain were not functional in *E. coli*.

Attempts were made to subclone the gene so that it was no longer under the control of the *lac* promoter. As the gene was subcloned so that it was no longer under the

control of the vector promoter, the level of expression became very low, and therefore, further studies on the regulatory aspects of melanin formation were not continued. There are several possible reasons for the lack of expression of the melanin gene from its own promoter in *E. coli*. The *A. chroococcum* promoter and regulatory sequences controlling polyphenol oxidase expression may not be recognized by *E. coli*. The cloned insert may lack an additional *Azotobacter* product which is required for transcription of the gene from its own promoter. Another possibility is that the cloned gene lacks a promoter altogether. However, this last possibility could be ruled out since initial screening of clones harbouring the *ppo* gene in an orientation which favours expression from its own promoter did show low levels of brown pigmentation and the gene is expressed in *A. vinelandii* most probably from its own promoter and not the *lac* promoter of *E. coli*, as was demonstrated using the mutated *lac* promoter upstream of the cloned gene. DNA sequencing of the *ppo* gene and its upstream sequences should provide more insight into the promoter sequence and the possible role of an activator element in regulating its expression.

The melanin locus of *Streptomyces antibioticus* has been cloned and sequenced (Katz et al. 1983; Bernan et al. 1985). It contains two open reading frames, which encodes the tyrosinase (835 bp; *mel*C2) and an upstream ORF438 (438 bp; *mel*C1) which are co-transcribed from the same promoter (della-Cioppa et al. 1990). The ORF438 is thought to be functional as an activator acting in *trans* and may be involved in tyrosinase secretion or as a metallothionein-like protein which delivers copper to the apotyrosinase (Lee et al. 1988). The cloned *Azotobacter* gene is on a 1.87 kb fragment of DNA and the requirement for a sequence like ORF438 for activation of the enzyme cannot be ruled out since copper is an essential requirement for enzyme activity. In *Streptomyces*, tyrosinase is secreted out into the growth medium and the majority of the melanin production takes place extracellularly (Lerch and Ettlinger, 1972; Bernan et al. 1985). In the *Azotobacter*, polyphenol oxidase activity is intracellular and the melanin is cell-associated (Chapter 3; Shivprasad and Page, 1989). However, with the cloned gene in *E. coli*, melanin accumulated extracellularly, and polyphenol oxidase activity was intracellular. Similar results were obtained with the tyrosinase gene of *Streptomyces* cloned in *E. coli*, where melanin accumulated extracellularly (della-Cioppa et al. 1990). della-Cioppa et al. (1990) speculate that DOPA or soluble melanin oligomers which are formed due to enzyme activity diffuse away from the bacterial colonies into the medium where they undergo further oxidative polymerization which is non enzymatic.

In the original melanin producing clone and its subclone in pLAFR3, the production of melanin was constitutive, being evident in 18 hours of growth and in

medium containing 20 µM copper sulphate. There was no requirement for IPTG in the growth medium, even though the gene was being transcribed from the *lac* promoter of the vector. This could be due to the presence of low levels of inducer in LB medium which are sufficient to induce the *lac* promoter. This is in contrast to the studies of Tseng et al. (1990), where they cloned the *Streptomyces antibioticus* tyrosinase gene into pMTL23. Optimum production of melanin in *E. coli* harbouring pIF413 or pIF415 required the introduction of IPTG into the growth medium and high copper levels (0.2 mM). Lower levels of copper resulted in delayed and weaker pigmentation (Tseng et al. 1990). In the study by della-Cioppa et al. (1990), the tyrosinase gene and ORF438 were cloned into a plasmid containing the T7 promoter. The *mel* gene was transcribed from the T7 promoter after heat shock at 42°C of the *E. coli* host strain which produced the T7 RNA polymerase. Maximum melanin production required 24-48 hours incubation at 30°C following heat induction.

On subcloning the *Azotobacter ppo* gene in multicopy pUC vectors, IPTG and higher copper levels were required for induction of tyrosinase, although in the absence of IPTG, melanization was evident after 48 hours with pMASS3. Since *E. coli* strain MV1193 contains the $lacI^q$ gene (Jeffrey Vierra, personal communication), and the plasmid is in multiple copies, IPTG was required for accelerating melanin production when the gene was cloned into pUC vectors.

It is not known why the cloned *ppo* gene failed to demonstrate activity on analysis by polyacrylamide gel electrophoresis. Since activity was observed in cell-free extracts by the tube assay and the cells produced melanin which diffused into the surrounding medium, it is possible that the conditions used during electrophoresis inactivated the polyphenol oxidase protein. Analysis of the polyphenol oxidase of *A. chroococcum* strain184 by non-denaturing polyacrylamide gel electrophoresis has identified the molecular weight of the enzyme complex to be 111K and 127K daltons (Chapter 3). This is similar to the size of mushroom tyrosinase tetramer (Jolley et al. 1969). Further purification of the polyphenol oxidase from *A. chroococcum* should give more insight to its subunit structure, K_m values for different substrates and its homology with other tyrosinases.

Tyrosine analogs such as N-acetyl-L-tyrosine and L-tyrosine ethyl ester gave pigment shades in *E. coli* which ranged from yellow to reddish brown as reported by della -Cioppa et al. (1990) as well. These modified melanins could be useful in developing color-modified melanins (della-Cioppa et al. 1990). Due to the reactivity of the quinone groups present in the melanin polymer, different compounds can be

incorporated into the pigment within the cell, resulting in the formation of a heteropolymer.

Melanins, due to their absorptive capabilities of electromagnetic radiation are potential material for applications in topical sunscreens as photoprotective agents. Another application of melanin is as a coating for plastics in order to delay photodegradation (Bialy, 1990). Presently, the availability of melanin is limited as squid ink sacs, bovine retinas or the chemical oxidation of DOPA (Bialy, 1990) are the only sources. Thus, since there is a growing interest in the use of natural materials and colours in the cosmetic industry, della-Cioppa et al. (1990) are looking into the large scale production of melanin by fermentation using the cloned gene in order to produce large amounts of melanin-based products with useful drug and cosmetic application. Thus the application of melanins is a growing field and due to the limited availability from natural sources, the ideal route would be to overproduce it by fermentative means as has been done with so many pharmaceutically important products.

In another application of expression of *mel* genes on plasmids, Altenbuchner (1988) and Tseng et al. (1990), describe the usefulness of melanin-expression cloning vectors in Gram negative bacteria. The plasmids constructed by Altenbuchner (1988) and Tseng et al. (1990) which harbour the *mel* locus of *S. antibioticus* under the control of the *lac* promoter have broad host ranges. Tseng et al. (1990) explain the usefulness of the *mel*C operon of *Streptomyces antibioticus* as an expression marker in *E. coli* and other Gram negative bacteria. The assay substrates are relatively inexpensive and there is no requirement for mutant host strains as is required for systems using the *lacZ* marker, which require *lac* mutant hosts. pIJ702 which harbours the *mel* operon of *S. antibioticus* has been used in the isolation of clones carrying components of the glycerol utilization operon and the undecylprodigiosin gene cluster of *Streptomyces coelicolor* (Katz et al. 1983). In our study, the *ppo* gene cloned into pLAFR3, the broad host range cloning vector (Staskawicz, et al. 1987) was transferred from *E. coli* to *A. vinelandii*. Cosmid pLAFR3 is also maintained in other Gram negative organisms like *Pseudomonas* species. Complete restriction mapping and nucleotide sequencing of the *Azotobacter ppo* gene cloned in *E. coli* would provide further details of the possible restriction endonucleases which could be used for insertional inactivation of the *ppo* gene.

de Bruijn & Lupski (1984) have reviewed the properties of Tn5 which make it useful for *in vivo* mutagenesis of cloned DNA sequences. Tn5 has been shown to be a useful tool to study bacteria whose genetic systems are usually not well developed (Cookson et al. 1990). In addition to having a low insertional specificity, the physical mapping of insertion mutations is facilitated by the presence of several restriction sites in

Tn5. Tn5*tac*1 has the added advantage of allowing exogenous control of gene expression with IPTG (Cookson et al. 1990). Tn5*tac*1 transposon contains a strong outward-facing promoter P_{tac}, a *lacI*^{q} repressor gene and a selectable kanamycin-resistance gene. Since transcription from P_{tac} is repressed by LacI protein, IPTG is required for induction. Therefore, when IPTG is absent, the mutation generated by Tn5*tac*1 is conditional since the mutation is polar on distal gene expression. However, when IPTG is added, it directs the transcription from these genes (Cookson et al. 1990). Using λ::Tn5*tac*1, mutants in melanin formation were obtained in *E. coli*. These mutant clones had Tn5*tac*1 inserted into the *Azotobacter* polyphenol oxidase structural gene (non-producers) or in the promoter (induced by IPTG), in this case, the *lac* promoter of the vector. Attempts which were made to replace the wild type *ppo* gene in *Azotobacter chroococcum* with the mutant structural gene by homologous recombination were not successful. Alternative methods will involve using the technique of electroporation as described by Trevors & Starodub (1990) where they have used this process for *A. vinelandii*.

It will be interesting to isolate a catechol-producing clone from the *A. chroococcum* strain 184 genomic library. These results with the cloned *ppo* gene suggest that it is catechol production that is ammonium regulated (Shivprasad & Page, 1989; Chapter 3) and since its regulation appears to be similar to the *nif* genes this aspect should prove very interesting to study. Although several schemes for screening for catechol production were tried in the present investigation (data not shown), none of them proved to be practical.

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CHAPTER 5

General Discussion

5.1 MICROAEROPHILIC CHARACTERISTICS AND RESPONSE TO OXIDATIVE STRESS

Strain 184 has demonstrated characteristics quite unlike those of the type strain *A. chroococcum* ATCC 9043 (Page, 1986; Page, 1987). Its requirement for sodium and iron for growth differs from the characteristics of organisms of this genus which demonstrate growth under iron-limited conditions, in response to which they produce siderophores (Page and Huyer, 1984; Page and von Tigerström, 1988). Strain 184, on the other hand demonstrates a minimal iron requirement for growth and hydroxamate production (Page, 1987). This quality resembles that of the microaerophilic magnetic bacteria *Aquaspirillum magnetotacticum* (Paoletti and Blakemore, 1986).

The lack of detectable catalase and the sensitivity to hydrogen peroxide and toxic oxygen radicals generated in culture media are similar to the characteristics of other microaerophiles (Hoffman et al. 1979; Padgett et al. 1982). This was followed by the observation of the presence of an initial band of growth at lower oxygen concentrations in semisolid medium and nitrogen-fixing conditions. The growth moved to a more aerobic environment as the cell density increased suggesting the preference for a microaerobic environment. This microaerobic growth preference of strain 184 further confirms the dissimilarity between this organism and the type species of this genus.

Further analysis of the oxygen requirement of the organism demonstrated the requirement for low oxygen tensions for growth and nitrogen-fixation which resembles the oxygen requirements of *Azospirillum* species (Day and Dobereiner 1976). Strain 184 demonstrated a preference for 10% O_2 in the presence of pyruvate as carbon source, and 16-18% O_2 with carbohydrates for maximum nitrogenase activity. Under microaerobic conditions the organism demonstrated a preference for organic acids as carbon source, quite like the carbon source requirements of *Azospirillum* species (Martinez-Drets et al. 1984). Drozd and Postgate (1970) have demonstrated the ability of *Azotobacter chroococcum* NCIB 8003 to grow and fix nitrogen at both low and high oxygen tensions, although higher efficiency of nitrogen fixation is obtained at low oxygen. Strain 184 demonstrates a preference for low oxygen tensions for maximum nitrogenase activity.

The inability to cope with toxic oxygen intermediates as demonstrated by the sensitivity to hydrogen peroxide and paraquat and the inability to grow on laboratory media, particularly when illuminated, unlike other nitrogen -fixing organisms which are similarly stressed, warranted an investigation into the mechanism of aeroadaptation of this strain. Molecules which bind iron and prevent it from participating in the Fenton reaction are thought to be involved in protecting a cell from the damaging effects of the hydroxyl radical (Gutteridge, 1987). Catechol, which is such a molecule, was observed in the culture supernatants of nitrogen-fixing cells. The catechol so formed was polymerized to a melanin. Production of catechols such as 2,3-Dihydroxybenzoic acid and 3,4-Dihydroxybenzoic acid have been reported from other species of the family *Azotobacteraceae*, (Page and Huyer, 1984; Collinson et al. 1987) although catechol formation has not been reported. Catechol formation was not repressed by iron in strain 184, but its production was accelerated by iron and high aeration. Under these conditions, the oxidative damage in a cell would be high due to hydroxyl radical formation by the Fenton reaction. High iron also accelerates hydroxylation of aromatic molecules (Halliwell, 1978), and therefore it appears as though the production of catechol is a response to oxidative stress in the cell. Charcoal, which is a free-radical scavenger enhanced the aerotolerance of this organism, as indicated by the improved recovery on plates containing charcoal, just as it has been demonstrated to do with several microaerophiles (Hoffman et al. 1983). Charcoal inhibited catechol and melanin formation although it did not enhance the nitrogen-fixing ability of the organism. DL-histidine, a hydroxyl radical trap (Hoffman et al. 1983), also inhibited catechol formation. Since aromatic molecules can be hydroxylated by hydroxyl radicals in a non-enzymatic process (Richmond et al. 1981) the production of catechol could be a mechanism of eliminating toxic oxygen radicals such as hydroxyl radicals from a cell, whereby a phenolic molecule is hydroxylated to produce catechol. Alternatively, since iron-binding catecholic compounds like norepinephrine are suggested to play a role as oxygen radical scavengers (Krieg and Hoffman, 1986) the catechol in the cell could itself be the free radical scavenger as has been demonstrated by the molecule Tiron (Miller and Rapp, 1973) or as mentioned earlier, it is an inhibitor of the Fenton reaction due to its iron-binding ability. It is also possible that both these systems viz., formation of catechol and the catechol formed play a role in protection of strain 184 from oxidative stress.

Interestingly, NH_4^+ repressed catechol formation at concentrations which also repressed nitrogenase in this organism. In the presence of nitrate and urea as well, catechol formation was repressed. Under nitrogen-fixing conditions, an organism is

more oxygen stressed than in the presence of ammonium. This is because of the presence of oxygen labile components of the nitrogenase system, such as the enzyme nitrogenase itself and the electron carriers, the flavodoxins (Robson and Postgate, 1980). In order to protect these components, organisms belonging to the genus *Azotobacter* increase their respiratory rate (Robson and Postgate, 1980), resulting in the production of more toxic oxygen radicals in the cell. Thus, catechol formation under nitrogen-fixing conditions correlates well with the intracellular oxidative stress conditions. The response of nitrogenase to methionine sulfoximine, a glutamine analog which derepresses nitrogenase in the presence of ammonium, was similar to the response shown by the catechol-forming system in strain 184. This suggests a similar regulatory mechanism for the two systems.

Melanin formation in organisms is known to be in response to stress conditions (Bell and Wheeler, 1986) and there has been convincing evidence for the role of melanin in protecting membranes from hydroxylation and therefore from damage (Sakina et al. 1980; Scalia et al. 1990). Melanin is formed due to the oxidative polymerization of phenolic compounds by a polyphenol oxidase (tyrosinase). Melanins are thought to inhibit the Fenton reaction due to their iron-binding abilities (Pilas et al. 1988) and are also free radical scavengers which have pseudo-superoxide dismutase activity (Korytowski et al. 1986). In the presence of free radical traps, like benzoate and charcoal, melanin formation was suppressed. The presence of the pigment in the membrane of the organism, the oxygen sensitive sites in a cell, would be beneficial to an organism which has demonstrated the laek of an essential enzyme which forms part of the defense system against strong oxidants generated in a respiring cell. Thus melanin formation also appears to be a response to oxidative stress in strain 184. Both catechol and melanin, chemical protectants, could be an alternative to or supplement to the enzymatic defense system present in the cell under nitrogen-fixing conditions. This role for melanin has been suggested by Sichel et al. (1987) and Sichel (1988) and therefore this could be the mechanism by which strain 184 demonstrates aeroadaptive capabilities.

The polyphenol oxidase of the organism was analysed by non denaturing gel electrophoresis and two isozymes were identified with apparent molecular weight which were estimated to be 111K and 127K daltons, similar to that of mushroom tyrosinase (Jolley et al. 1969). Although melanin formation was not demonstrated under ammonium-grown conditions, polyphenol oxidase activity bands were observed even in the absence of copper in the growth medium. Thus the synthesis of the enzyme is not under negative control by ammonium as was initially speculated. Catechol formation could be regulated by ammonium at the genetic level, since it is the substrate for the

enzyme under nitrogen- fixing conditions. The interesting observations of catechol and melanin formation at the physiological level prompted an investigation of this aspect at the molecular level.

5.2 GENETIC STUDIES ON STRAIN 184

A genomic library of strain 184 was constructed using the broad host range cosmid cloning vector pLAFR3 (Staskawicz et al. 1987). A melanin-producing *E. coli* clone was isolated and the 20 kb insert in pLAFR3 (pMASS1) was subcloned as a 1.87 kb *Bam*HI-*Sst*I fragment into pUC119 (pMASS3). The production of melanin in the clone was dependent on the presence of tyrosine and copper in the growth medium, identifying the melanin as a DOPA-melanin which was not cell-associated, unlike the catechol-melanin which was cell-associated in nitrogen-fixing strain 184.

When the polyphenol oxidase gene was subcloned into the high copy number vector of the pUC series, the level of copper required to activate the enzyme was higher. This explained the effect of copper seen in strain 184 under ammonium-grown conditions. That is, due to the high total cell protein (which is more than nitrogen-fixing cells in the same time period), the amount of copper available to the enzyme, which is a copper-containing enzyme was lower. Thus, although polyphenol oxidase activity bands were observed in the cell-free extracts of ammonium-grown cells, the enzyme was not active *in vivo* until additional copper was added to the growth medium.

The production of melanin in the clone is regulated by the vector promoter, the *lac* promoter thus necessitating the presence of IPTG in the growth medium only to accelerate melanin formation. The presence of the gene's own promoter, an *Azotobacter* promoter, was confirmed due to the production of melanin in *Azotobacter vinelandii* when the cosmid containing the *ppo* gene was introduced into the organism by triparental mating. Tn5 mutants of the polyphenol oxidase structural gene in pLAFR3 have been obtained using the Tn5 derivative, Tn5*tac1* (Chow and Berg, 1988) with lambda phage as a vehicle for introducing the transposon. It is proposed to return the mutated gene back into strain 184 by homologous recombination to obtain a *mel*$^-$ mutant so that we may study the role of melanin in aeroadaptation.

The organism harbours a plasmid which has a size of 30-35 kb. The characteristics encoded by the plasmid have not been investigated, although the production of melanin, is a possibility, since melanin production is a plasmid encoded property in *Rhizobium* species (Hynes et al. 1988) and in some *Streptomyces* spp. as well (Gregory and Huang, 1964). The presence of a plasmid is thought to be the cause

of the difficulty in transforming organisms belonging to this species (Robson et al. 1984) of *Azotobacter*.

16S rRNA sequencing data (E.R.B. Moore, G.E. Fox, and P. Jurtshuk, Jr. Abstract # H-251, p. 196: Abstracts of the 90th Annual Meeting of the American Society for Microbiology, Anaheim, California, 13-17 May, 1990) accompanied by the available data on the diverse metabolic characteristics of strain 184 warrants its reclassification as a new species, *Azotobacter salinestris* species *novellus* (Page and Shivprasad, submitted for publication).

5.3 FUTURE STUDIES

The mechanism by which strain 184 adapts to aerobic conditions of growth would be interesting to investigate. This study has provided a starting hypothesis for the mechanism of aeroadaptation. The construction of a genomic library has provided a starting tool to isolate and identify various gene products that could be involved in protection from oxidative stress and also to study the cause of the sodium requirement of the organism.

The isolation of a melanin-producing clone provides an initial step into studying the role of melanin production in aeroadaptation. Further studies could involve sequencing the polyphenol oxidase gene of strain 184, including the promoter and upstream regions and studying the regulatory mechanisms involved in melanin formation. Since tyrosinases have shown such diversity in molecular size and sequence, except at the active site (C. Fuqua, personal communication), it would be interesting to compare the nucleotide and amino acid sequence of the polyphenol oxidase of this organism with that of other known sequences such as that of *Streptomyces* (Bernan et al. 1985). Once the sequence is known, an oligonucleotide probe can be constructed to determine the location of the gene, i.e. whether it is chromosomally-located or on the plasmid in strain 184. Sequencing of this gene and its regulatory region should also provide information about the cause of the reversible instability of melanin formation. This could either be due to a transposition event as has been demonstrated in *Streptomyces* species (Freeman et al. 1977) or due to plasmid involvement. The latter possibility could be eliminated due to the reversibility of the instability.

Since Tn5 mutants in the structural gene have been obtained, the mutated gene can be returned into strain 184 by homologous recombination, as described by Ruvkun and Ausubel (1981). This would provide a direct approach in determining the role melanin has in strain 184. Since so far, attempts to transform strain 184 have not been

successful, it is possible that electroporation could be a possible method of introducing foreign DNA into strain 184. This method has been used in the transformation of *Azotobacter vinelandii* (Trevors and Starodub, 1990). Until now, the role of melanin in bacteria has not been elucidated, although in fungi it is known to play a role in protection from hydrolytic enzymes (Bell and Wheeler, 1986). Studies *in vitro* have demonstrated that the role of melanin is in inhibiting lipid peroxidation of membranes and therefore protection from oxidative damage (Sakina et al. 1980; Scalia et al. 1990). Since strain 184 can be easily cultivated on laboratory media, it would be a good tool to use for studying the role of melanin, *in vivo*, particularly with the isolation of a *mel*⁻ mutant.

Recently, there has been an interest in the large scale production of melanin due to its free-radical scavenging properties and its role as a sunscreen. della Cioppa et al. (1990) have cloned the tyrosinase gene from *Streptomyces antibioticus* into *E. coli* with an intent on producing melanin on a large scale for use in the drug and cosmetic industry. The cloned polyphenol oxidase gene from strain 184 could serve the same purpose, particularly since it does not require any additional factors or treatments for melanin production except tyrosine and copper supplemented in LB medium. This increasing interest in melanin as a sunscreen is due to the global changes in the environment, as a result of which, we humans are being exposed to an increased dose of ultraviolet light. It is thought that fair skinned people will be more prone to skin cancers than darker skinned people and it is possible that della Cioppa et al. (1990) would like to exploit the free-radical scavenging properties of melanins for this purpose. della Cioppa et al. (1990) also report that the final aim of their project is to introduce the tyrosinase gene they have cloned, into plants. Bialy (1990) further emphasizes the use of melanin in the plastic industry for the production of plastics resistant to photodegradation. Thus it appears that the large scale production of melanin will be an important project for biotechnologists in the near future.

Since catechol formation in strain 184 was demonstrated to be ammonium regulated and is thought to play a role in aeroadaptation, it would be interesting to isolate a catechol-producing clone from the genomic library and studying its regulation. The similarity between regulation of catechol formation and the *nif* genes should provide an interesting subject to investigate. Further studies on catechol formation should be interesting and would throw some more light on the mechanism of aeroadaptation in this organism.

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